ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL CANCER INSTITUTE

FISCAL YEAR 1971
PART III

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

NATIONAL CANCER INSTITUTE

Fiscal Year 1971

PART III

RC
267
U5626
1971
pt.3

NATIONAL CANCER INSTITUTE

ANNUAL REPORT

EXTRAMURAL ACTIVITIES

July 1, 1970 through June 30, 1971

TABLE OF CONTENTS

		Page
I	Office of the Associate Director	1667
II	Program Analysis and Reporting Section	1673
III	Awards Review and Technical Administration Branch	1677
	A. Carcinogenesis Program	1679
	B. Cancer Biology Program	1683
	C. Cancer Epidemiology Program	1687
	D. Pharmacology Program	1689
IV	Clinical Investigations Branch	1695
	A. Cooperative Clinical Research Program	1695
	B. Leukemia Center (Platelet) Program	1699
	C. Protected Environment Program	1699
	D. Radiation Therapy Program	1701
	E. Immunology Program	1705
V	Special Programs Branch	1707
	A. Cancer Research Centers	1707
	B. Clinical Cancer Training Program	1709
	C. Graduate Research Training Program	1711
	D. Research Career Program	1715
	E. Fellowships Program	1719
VI	Cancer Special Program (Sloan-Kettering Institute)	1723
VII	Appendix I (Published Results of Scientific Work Supported by NCI)	1725
	A. Chemical Carcinogenesis	1726
	B. Viral Carcinogenesis	1733
	C. Cancer Biology and Biochemistry	1739
	D. Host-Tumor Interactions	1747
	E. Epidemiology	1754
	F. Diagnosis	1761
	G. Pharmacology	1768
	H. Therapy	1772
	I. References	1791
VIII	Statistical Summary of Awards	1805

NATIONAL CANCER INSTITUTE

ANNUAL REPORT

July 1, 1970 through June 30, 1971

TABLE OF CONTENTS

		Page
GENERAL LABORATORIES AND CLINICS		
Laboratory of Biochemistry		
Summary Report		1807
Project Reports:		
NCI-375	Homogeneity and Structure of Proteins	1815
NCI-333	Biochemical and Immunochemical Studies on Normal and Leukemic Leukocytes	1818
NCI-5203	Development of Immunochemical and Physical Methods for the Purification and Characterization of Immunocytes and Their Subcellular Components	1822
NCI-5202	Isolation, Fractionation, and Characterization of Native Nucleoproteins	1824
NCI-380	Deoxyribose Containing Compounds in Normal, Regenerating and Cancer Tissues	1827
NCI-5211	Initiation and Maturation Phenomena in the Replication of Mammalian DNA	1830
NCI-5205	Studies on Biochemical Mechanisms of Sensitivity and Resistance to Antimetabolites and Mechanisms for Control of Nucleic Acid Synthesis	1833
NCI-397	Studies Comparing the Structure, Properties and Metabolism of the Nucleic Acids and Nucleoproteins of Cultured Cells and of Tumor Derived from Them	1836
NCI-394	Studies on Isozyme Distribution in Various Transplantable Rat Hepatomas	1838
NCI-306	Studies on β-hydroxy-α-amino Acids	1840

Laboratory of Biochemistry (Cont'd) Page

NCI-366 Biosynthesis and Assembly of Intracellular Structural Components 1843

NCI-5210 Control of Specific Protein Synthesis in Animal Cells 1845

NCI-379 Ultrastructural Characterization of Immunoglobulins, Enzymes and Other Biomacromolecules 1849

NCI-5215 Biological and Ultrastructural Studies on Cell Cooperation in the Immune Response 1852

NCI-5212 Virus-cell Interaction of Oncogenic Viruses 1855

NCI-5213 Radioassay Techniques for Gas Producing Reactions 1858

NCI-5214 Mechanism and Regulation of RNA Synthesis in Eucaryotic Cells 1860

NCI-368 Studies on the Mechanisms of Mammary Tumor Regression 1864

NCI-5216 Role of Lysosomal Enzymes in the Regression Process of Mammary Tumors 1867

NCI-334 The Occurrence and Function of a Stress-modifiable Insulin: Anti-insulin Hormonal Complex Regulating Glucose Utilization in Cancer and Normal Cells, and Its Relation to Chemotherapy at Normal and Hyperthermic Temperatures 1869

NCI-330 Production of Healthier Cigarettes 1872

NCI-331 Anticancer Effects of Amygdalin and Derivatives 1874

Laboratory of Biology

Summary Report 1877

Project Reports:

NCI-3208 The Study of the Inheritance of Mammalian Proteins and Their Relationship to the Incidence of Tumors 1883

Laboratory of Biology (Cont'd) Page

NCI-3251 Testing for Possible Carcinogenic Action of Enovid in Mice 1886

NCI-3253 The Influence of Age and Sex Hepatocarcinogenesis Induced by Chemical Carcinogens 1888

NCI-3254 An Ultrastructural, Biochemical, and Immunological Characterization of Avian Myeloblastosis Virus (AMV) I. Isolation of Cores. II. Localization of the 4S RNA. III. Identification and Localization of Enzymatic Capacities 1890

NCI-3255 A Correlative Light and Electron-micrographic Study of Hepatocarcinogenesis Induced by Chemicals 1892

NCI-3256 Ultrastructure and Biology of Mammary Tumor Virus-host Cell Interactions 1894

NCI-3259 Host Genome-viral Relationship in Mammary Tumor Development in Mice 1896

NCI-3260 Studies of Mammary Tumors in Resistant Strain C57BL Mice 1899

NCI-3261 Chemical and Ultrastructure Studies on Cytoplasmic DNA 1901

NCI-3211 Studies, by Tissue Culture and Cell Culture Methods and by Amplifying Methods, of the Malignant Cell and of the Normal Cell from which It Arises; Studies on the Mechanism of Transformation of the Normal Cell into the Malignant or Cancer Cell; Studies on Characterization and Identification of Such Cells; Studies on Associated Significant Subjects 1902

NCI-3212 Proj. 1 Studies of Cell Nutrition and Metabolism in vitro 1906

1. To Test Ability of Cells to Grow in Completely Chemically Defined Medium
2. To Study Growth and Metabolism of a Neoplastic C3H/HeN Mouse Cell Lime with Special Emphasis on Vitamin B12, Folic Acid Interrelationships, and Nucleic Acid Precursors
3. To Determine the Effect of Fractions of Horse and Fetal Calf Serum on Growth of Freshly Explanted C3H Mouse Embryo Cells

Laboratory of Biology (Cont'd) Page

NCI-3212 Proj.1 (Cont'd) 4. To Test Ability of C3H Mouse Embryo Cells to Grow in Medium Containing Gelding Mare or Stallion Serum Obtained from a Commercial Source

NCI-3212 Proj.2 Cell Transformations in vitro 1910

NCI-3213 Proj.1 Carcinogenesis in vitro 1917

NCI-3213 Proj.2 Manifestations of Neoplastic Transformation in vitro 1922

NCI-3234 (a) Primary Biochemical Alterations Leading to Neoplastic Conversion 1927

NCI-3257 Factors Influencing the Growth and Neoplastic Conversion of Cells in vitro 1931
1. Influence of Glycine Peptides upon Cells in vitro
2. Efficiency of Commercially Available Peptones as Cell Growth Stimulants and/or Substitutes for Serum
3. Evaluation of some Organic Buffers for Cells in vitro
4. The Development of a Homeostatic Cell Culture System
5. Serum Hamster Cells Exposed to Benzpyrene (Refer to Serial No. NCI-3213, project 1)

Laboratory of Cell Biology

Summary Report 1937

Project Reports:

NCI-3200 Factors Influencing the Induction and Repression of Primary Tumors and the Growth and Inhibition of Transplantable Neoplasms. (Host and Tumor Cell Variables) 1941

NCI-3229 Chemical Structure of Immunoglobulin Molecules 1946

NCI-5552 Production and Analysis of Hybrid Somatic Cells: Their Application to Some Problems of Importance in Biology and Medicine 1950

NCI-5551 Development of in vitro Tests to Detect Tumor-specific Transplantation Antigens. Several Techniques Are Currently Under Development 1955

Laboratory of Cell Biology (Cont'd) Page

NCI-5556	Pathogenesis of Plasma Cell Neoplasia in Mice; Studies on Antigen-binding Myeloma Proteins; Genetic Basis of Antibody (Immunoglobulin) Diversity; Screening of Human Myeloma Proteins for Antigen-binding Activity	1957
NCI-5554	A. Pathogenesis and Pathophysiology of Plasma Cell Neoplasms in Mice and Characterization of Their Immunoglobulin Products B. Characterization and Measurement of Soluble Antigens Associated with Human Neoplasms	1965
NCI-3222	Genetic and Biochemical Mechanisms Controlling Protein Synthesis in Well-differentiated Neoplastic Cells, Mouse Plasma Cell Tumors	1968
NCI-5523	Spontaneous Association of Enzyme Subunits	1972
NCI-5531	Nature and Aggregation of Subunits of Enzymes	1975
NCI-5533	Fine Structure in the Fluorescence Spectrum of L-asparaginase	1977
NCI-5534	Chemical Modification of L-asparaginase	1979
NCI-5535	Properties of L-asparaginase Derivatives	1981
NCI-5503	Properties of Mouse Leukemia Virus RNA	1983
NCI-5521	Separation and Characterization of Rat Liver Mitochondrial DNA Strands and of Other Biopolymers in Density Gradients	1985
NCI-3101 (d)	Nucleic Acid Homologies between Normal Rat Liver and Morris Hepatoma 5123	1988
NCI-5529	SV40 Tumor-specific Transplantation Antigens (TSTA)	1991
NCI-5536	SV40 Tumor-specific Transplantation Antigen Expression in Cells Infected or Transformed by Mutants of SV40 Virus	1993
NCI-5537	Cr^{51} Release Micro Assays for Measurement of Histocompatibility Antigens	1995

Laboratory of Cell Biology (Cont'd) Page

NCI-5526 Glycolipids in Normal DNA Virus Transformed and Spontaneously Transformed Tumorigenic Mouse Cell Lines 1997

NCI-4833 Electron Microscope Studies of Normal, Malignant and Virus-infected Cells and Cell Fractions 2002

NCI-5550 Studies on Murine Leukemia and Sarcoma Viruses 2006

Laboratory of Molecular Biology

Summary Report 2011

Project Reports:

NCI-8000 Regulation of Gene Expression in E. coli by Cyclic AMP 2013

NCI-8001 Role of Cyclic AMP in the Regulation of Growth and Morphology of Transformed and Normal Fibroblasts 2018

NCI-8002 Analysis of the Fusion of XC Cells Induced by Homogenates of Murine Leukemia Virus-infected Cells and by Purified Murine Leukemia Virus 2020

NCI-8003 Integration and Excision of Phage λ 2022

NCI-8004 Control by Lambda Genes of the Expression of Bacterial Genes 2024

Laboratory of Pathology

Summary Report 2027

Project Reports:

NCI-500 Histogenesis and Pathology of Induced and Spontaneous Tumors of Laboratory Animals 2033

NCI-501 Carcinogenesis in the Cheek Pouch of Hamsters or in the Esophagus of Hamsters and Rats: A Search for Factors in the Environment that may be Related to Cancers in Man 2038

Laboratory of Pathology (Cont'd) Page

NCI-501 (a)	Geographic Pathology Field Study on Uterine Cancer in Negro Women in New York City and the Washington, D. C. Metropolitan Area	2040
NCI-501 (b)	A Review of the Scientific Literature on the Subject of "Cancer in Man at the Site of Prior Benign Lesion of Skin or Mucous Membrane"	2041
NCI-501 (c)	Effects of Gambier, an Ingredient of the Betel Quid, Administered to Mice by Various Routes	2042
NCI-501 (g)	Report on Histologically Diagnosed Cancers in Indians of the United States, 1950 to 1966	2043
NCI-504	An Electron Optical Investigation of Normal and Pathologic Tissue	2044
NCI-504 (b)	An Investigation of the Collagenous Connective Tissues and Elastin	2046
NCI-505	Evaluation of Potential Pulmonary Carcinogens, Asbestos, Tobacco, Silicates and Heavy Metals and their Relation to the Physiological Status of the Lung	2048
NCI-514	Induced and Spontaneous Neoplasms in Laboratory Animals. Factors that Influence their Incidence and the Chemical Changes Induced by the Neoplasms	2056
NCI-516 (c)	Viral Carcinogenesis. The Murine Sarcomagenic Viruses: Problems of Immunity, Resistance, and Pathogenesis and their Relation to Cell Structure	2059
NCI-519	Induction of Neoplasms, Selection of Transplantable Strains of Tumors, and Study of Tumor and Host Chemistry	2061
NCI-521 (a)	Investigations of Mechanisms of Tumor Induction by Polyoma Virus in Mice	2063
NCI-538	A Study of Phylogenetic Aspects of Neoplasia	2067

Laboratory of Pathology (Cont'd) Page

NCI-579 The Action of Methylazoxymethanol on Rat Mesonephros and Metanephros in Organotypic Culture 2071

NCI-560 Electron Probe and Scanning Electron Microscope 2073

NCI-561 Collaborative Research and Other Activities 2075

NCI-527 Immunocytology and Physiopathology of Cancer 2076

NCI-852 Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems 2080

NCI-854 Experimental Studies on Carcinogenesis, Endocrine Factors and Cytogenetic Characters 2084

NCI-563 Studies of Mechanisms of Action in Tumor Immunology 2088

NCI-569 Control of Lymphoma Development in Mice after Dilantin Treatment 2091

NCI-572 Immunological Lymphoma Development 2092

NCI-574 Maturation of Lymphoreticular Tissue as Affected by Antigenic Stimulation of the Fetus 2094

NCI-575 Histopathologic Study of Graft-Versus-Host Reaction in Human and Animal Bone Marrow Allograft Recipients 2095

NCI-576 Histopathologic Study of Side Effects of Cancer Chemotherapy with Special Attention to the Immunosuppressive Effect 2097

NCI-517 Pathological Technology Section 2099

NCI-853 The Surgical Pathology and Postmorten Sections, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all Institutes in those investigations which involve the use and study of human pathological material. The staff of the Surgical Pathology and Postmortem Section also carries on independent and collaborative research. 2101

Laboratory of Pathology (Cont'd) Page

NCI-857	Interstitial Pulmonary Emphysema: Acute and Chronic Morphological and Physiological Changes Induced by Elevation of Intratracheal Air Pressure	2112
NCI-858	Induction of Ovulation by Gonadotropic Hormones: A Clinico-pathologic Study	2115
NCI-859	Renal Biopsy in Lupus Nephritis: A Statistical Analysis of Variability of Disease at Different Sites within Single Kidneys	2116
NCI-866	Ultrastructural Studies of Primary Liver Cancer	2117
NCI-867	Hodgkin's Disease Involving the Lung	2118
NCI-871	Light and Electron Microscopic Studies of Tumors and Cell Lines Associated with H. saimiri	2119
NCI-555	Ultrastructural and Autoradiographic Studies of Arbovirus Replication	2120
NCI-555 (a)	Ultrastructural and Biological Studies of Effects of an Antiviral Drug on Poxvirus Infections	2122
NCI-577	Ultrastructural and Biologic Studies of Endoreticular Tubules in Neoplastic and Virus-infected Cells	2124
NCI-861	Ultrastructural, Histochemical and Biological Studies of Hormonally Active Tumor Cells	2126
NCI-863 (a)	Ultrastructural Studies of Human Surgical and Cytology Specimens	2128
NCI-863 (b)	Ultrastructural and Biologic Studies of Cells Infected with Herpes-group Viruses	2129
NCI-524	Studies on Interferon Production and Action	2131
NCI-531	In Vitro Studies of Human Malignant Lymphomas, Sarcomas and Retinoblastoma	2134
NCI-532	Studies of Oncogenic DNA Viruses	2136

Laboratory of Pathology (Con'd) Page

NCI-554 Studies on Replication of an RNA Virus 2138

NCI-567 Studies on a Sarcoma Virus 2140

NCI-578 Studies of Molecular Mechanisms Controlling the Differentiation of Normal and Malignant Cells 2142

Laboratory of Physiology

Summary Report 2145

Project Reports:

NCI-905 Iodide-Concentrating Mechanism of the Thyroid Gland 2149

NCI-906 Production and Properties of Transplantable Thyroid Tumors 2151

NCI-928 Intracellular Colloid Droplets 2152

NCI-919 Urinary Excretion Patterns in Leukemia 2153

NCI-925 Protein Synthesis and Its Control in Normal and Tumor Cells 2156

NCI-946 Role of Hemin in the Control of Globin Synthesis 2159

NCI-944 (a) Total Metabolism 2161

NCI-944 (b) Mathematical Programming 2164

NCI-916 Characterization and Comparison of the Nucleic Acids, Nucleoproteins and Related Components of Normal and Malignant Tissues 2167

NCI-945 Study of the Factors Regulating the Initiation of Specific Protein Synthesis During Embryonic Development 2169

NCI-948 Control of Ribo- and Deoxyribonucleotide Synthesis 2175

NCI-936 Time-dependent Effects of an Initial Treatment on Tolerance to Subsequent Treatments; Radiation and Vinblastine, with an Application to Experimental Therapy 2178

Laboratory of Physiology (Cont'd) Page

NCI-941 Immunogenetic Factors Affecting Bone Marrow Transplantation in Irradiated Mice: Modification of the Functional Capacity of Bone Marrow 2180

NCI-942 The Effects of Ionizing Radiation on Macromolecules of Biological Importance 2183

The Endocrine Evaluation Branch and Breast Cancer Task Force Program

Summary Report 2191

ASSOCIATE SCIENTIFIC DIRECTOR FOR CLINICAL RESEARCH

Project Reports:

NCI-15-000 Screening 2199

NCI-15-001 Screening 2200

NCI-15-002 Emergency Admissions 2201

Dermatology Branch

Summary Report 2203

Project Reports:

NCI-3607 Tissue Culture Studies of Human Lymphocytes 2209

NCI-3612 Mycosis Fungoides: Prognostic Features, Autopsy Findings, and Response to Therapy 2213

NCI-3613 Extraction, Isolation and Characterization of Keratohyalin Granules 2215

NCI-3614 Psoriasis Vulgaris: Treatment with Topical Nitrogen Mustard 2219

NCI-3617 1) Electron Microscopic Study of the Ultrastructure of Darier's Disease. 2) Study of the Development of Giant Melanin Granules in the Beige Mouse and the Study of a 'Double Mutant' Effect on Melanin Granule Formation in the Beige-Ruby. Beige-Pink and Beige-Albino Mice. 3) Continuation of the Study of Sezary Cells in Skin Lesions. 4) Study of the Ultrastructural Defect Caused by a Specific Exotoxin Formed by Group II Staphlococci in the Skin of Newborn Mice, a Model for Ritter's Disease of Infants 2221

Dermatology Branch (Cont'd)		Page
NCI-3618	Generation of Cyclic-AMP in the Urine of Patients with Basal Cell Nevus Syndrome (BCNS)	2225
NCI-3622	Viral Etiology of Pemphigus	2227
NCI-3623	Evaluation of Various Elastic and Collagen Tissue Diseases	2229
NCI-3625	Epidermal Macromolecular Synthesis in Newborn Rat, and Normal and Diseased Human Skin: An Electron Microscopic, Autoradiographical Study	2231
NCI-3626	In Vitro Carcinogenesis Utilizing Dimethylbenzanthracene and Other Solvents	2234
NCI-3629	The Use of Osmium Zinc Iodide (OsO_4 - ZNI_2) as a Special Stain for the Golgi Apparatus and Other Subcellular Organelles	2236
NCI-3630	Vitamin A Acid Induced Mucous Metaplasia of 13 Day Embryonic Chick Metatarsal Skin in Organ Culture: An Histochemical and Electron Microscopic Study	2238
NCI-3631	Treatment of Skin Cancer with Topical BCNU, and CCNU	2241
NCI-3632	Toxic Epidermal Necrolysis and Graft-Versus-Host Reaction	2244
NCI-3633	Treatment of Psoriasis with Topical CCNU. (IND-7003)	2246
NCI-3634	The Role of DNA Repair Mechanisms in the Etiology of Cancer	2248
NCI-3635	Investigation of Lysosomal Activity in Epidermolysis Bullosa Simplex (EBS)	2250
NCI-3636	Susceptibility of Fibroblasts from Xeroderma Pigmentosa Patients to Oncogenic Virus Transformation In Vitro	2252

Immunology Branch — Page

Summary Outline and Summary Report 2255

Project Reports:

NCI-5000 (c) In Vitro Biosynthesis of Immunoglobulins 2263

NCI-5001 (c) NCI Immunoglobulin Reference Center 2266

NCI-5002 (c) Genetic, Structural and Functional Characteristics of Human Immunoglobulins 2270

NCI-5003 (c) Cellular Immunity I 2276

NCI-5004 (c) Regulation of Growth and Biosynthetic Activity of Cells of the Immune System 2279

NCI-5005 (c) Investigations of Malignant Lymphocytic and Plasmacytic Diseases 2282

NCI-5006 (c) Transplantation Antigens 2284

NCI-5008 (c) Transplantation of Hematopoietic Cells in Humans 2289

NCI-5009 (c) Immune Response to Tumor Specific and Other Cell Surface Antigens 2294

NCI-5011 (c) Basic Immunological Studies in Hematopoietic Cell Transplantion 2299

NCI-5012 (c) Study and Reconstitution of Patients with Deficient Immune Systems 2302

NCI-5014 (c) Lymphocyte Stimulation: The Chemical Basis of Antigen Recognition and Gene Activation 2304

NCI-5015 (c) The Immune Response to Primary Cancers 2307

NCI-5016 (c) Cellular Immunity II 2310

Metabolism Branch

Summary Report 2315

Project Reports:

NCI-4002 (c) Studies of Physiology and Pathophysiology of Plasma Protein Metabolism 2323

Metabolism Branch (Cont'd) Page

NCI-4003 (c) Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria 2328

NCI-4004 (c) Studies of Amino Acid Transport and Protein Synthesis 2331

NCI-4009 (c) A Study of the Factors Controlling Red Blood Cell Synthesis and Destruction 2334

NCI-4013 (c) Studies of Calcium Metabolism in Malignant Disease and Metabolic Disease of Bone 2338

NCI-4014 (c) Studies of the Immune Response in Normal and Pathological States 2340

NCI-4015 (c) Studies of the Development and Function of Humoral and Cellular Mechanisms of Host Defense 2345

Radiation Branch

Summary Report 2353

Project Reports:

NCI-958 (c) The Radiotherapeutic Management of Hodgkin's Disease 2355

NCI-6052 (c) Evaluation of Prophylactic Chemotherapy in the Management of Ewing's Sarcoma 2358

NCI-6054 (c) Radiotherapeutic Management of Lympho-proliferative Diseases (Chronic Lymphocytic Leukemia and Lymphocytic Lymphomas) 2360

NCI-965 (c) Radiation Therapy of the Hypothalamic-Pituitary Lesion of Hand-Schuller-Christian Disease 2362

NCI-964 (c) Use of Systemic Chemotherapy as an Adjuvant to Radiation Therapy 2364

NCI-6050 (c) Computer Applications in Radiation Therapy 2366

NCI-961 Biologic Effects of Radiation on Mammalian Tumor Cells 2367

NCI-963 Effects of Immunologic Manipulation on Radiation Carcinogenesis and Radiation Response of Tumors 2368

Radiation Branch (Cont'd) Page

NCI-650 (c) Service Radiation Therapy 2370

NCI-684 Non-Clinical Service Irradiation 2371

NCI-947 Development, Fabrication and Maintenance of Specialized Equipment for Radiation Branch, Laboratory of Physiology, and Several Other Laboratories in NCI 2374

Surgery Branch

Summary Report 2377

Project Reports:

NCI-3800 Removal of Cancer and Other Tissues for Histologic Biochemical and Other Studies as Required by Scientists and Other Investigators for Correlated Studies 2389

NCI-3801 Evaluation of Cancer Surgery as to its Effectiveness and Safety in Providing Definitive and Therapeutic Care of Cancer Patients 2404

NCI-3805 The Experimental Studies of Mechanisms of Metastases 2437

NCI-3806 Surgical Physiology and Metabolic Changes Associated with Extensive Cancer Surgery 2462

NCI-3813 Surgical Endocrinology 2473

NCI-3816 Cryobiology 2478

NCI-3817 The Subcellular Mediation of Immune Responses to Tumor Antigens 2488

NCI-3818 Malignancy Associated Cellular and Serum Abnormalities (Formerly "The Relationship of Lymphocytic Activity and the Immune Mechanism") 2497

NCI-3819 Study of Cancer Immunology and Immunotherapy in Laboratory Animals 2506

NCI-3820 Cellular Immunity to Tumor Specific Transplantation Antigens 2518

Surgery Branch (Cont'd) Page

NCI-3808 Study of Host Factors Associated with Implantation and Growth of Cancer 2526

NCI-3815 Tissue and Organ Transplantation 2545

ASSOCIATE DIRECTOR, EXTRAMURAL ACTIVITIES
NATIONAL CANCER INSTITUTE

The Extramural Activities area, as an integral part of the total program of the National Cancer Institute, is responsible for the planning, development, evaluation, and administration of national and international programs for support of cancer research and training through Federal grants and awards. The Office of the Associate Director for Extramural Activities also serves as the focal point for the implementation of policies governing the review and management of contracts.

During the past year a program director was appointed to administer the important area of diagnostic research and prevention, an area long recognized as one requiring additional emphasis. Recent leads in the biological sciences applicable to diagnostic research and development in instrumentation and computer technology prompted the initiation of a program in this area. This will permit the NCI to stimulate research and training programs in the area of diagnosis and prevention.

An interesting development has been the exploration of a new technique of employing the grant mechanism in the establishment of targeted programs in the areas of colorectal cancer and bladder-prostate cancer. This technique involves a departure from the usual practices of traditional operations of support of biomedical research while retaining the dual review aspects of grant supported activities. After reviewing the detailed plans, the National Advisory Cancer Council will recommend a lump sum of money, adequate for the approved program. Individual grants will then be awarded upon recommendations made to the NCI by the working bodies established to plan and operate those studies. A special advisory committee will review the practices of the working bodies and will report periodically to the Council on the progress of the research and the quality of the review processes employed.

A new program director was appointed to administer the Immunology Program, which had been instituted in December 1969. This will operate in the Clinical Investigations Branch. The Chemotherapy Program, also in this branch, was activated in April 1970, with the appointment of a program director. The successful direction of the Radiation Therapy Program in fiscal year 1970 by a guest scientist on sabbatical leave led to the appointment of another such scientist for fiscal year 1971. The visiting scientist for this year is Dr. Patrick J. Cavanaugh, Chief, Division of Radiation Therapy, Duke University.

Council Presentation

The presentation at the November Council meeting of a series of charts reflecting trends in research grant awards from fiscal years 1962 through 1970 provoked considerable discussion. Concern was expressed regarding the funding of young investigators in the light of budget restrictions, and the effect of reductions on cancer programs in general. It was thought that current constraints might serve to discourage new investigators from applying for grants. As a consequence of these concerns and in support of further discussion at the March Council meeting, various data were collected and additional charts and tables

were prepared to indicate the trend of research grant funding for the fiscal years 1967 and 1970. These years represent the zenith and nadir years, respectively, in number of grants awarded and amounts funded.

Funding Comparisons, Fiscal Years 1967 and 1970

As to the effect of reductions in the past few years on cancer programs, the data indicate that while a greater percentage of support was allocated for program projects and centers than previously, the overall total dollars remained relatively constant and this constant relationship prevailed for the first, second, and third ten institutions grouped in order of total NCI support:

Institutions Funded	Fiscal Year 1967	Fiscal Year 1970
	(Dollars in thousands)	
Number of institutions funded	328	273
Number of grants awarded	1,828	1,509
First ten institutions	$29,542	$29,769
Second ten institutions	12,928	12,694
Third ten institutions	8,192	9,102
Sub-total	$50,662	$51,565
Remaining institutions	33,445	32,573
Total	$84,107	$84,138

Of the top 30 institutions funded in fiscal year 1967, five were no longer in the top 30 group funded in fiscal year 1970, having been replaced by five different institutions.

Age Level Funded

The study revealed that in fiscal year 1970 the median age of funded principal investigators was lower than in fiscal year 1967. The percentage of scientists aged 25-35 supported was greater in 1970 than in 1967. It was further indicated that the percentage of renewal applications supported in the 30-35 age group in fiscal year 1970 was 23; in the 36-40 age group, the percentage was 18. In the renewal grant area also, a higher percentage of beginning investigators (25-30 age group) were funded in fiscal year 1970 than in fiscal year 1967.

Budget

In fiscal year 1971 the appropriation for the Extramural Activities area was 18.5 percent higher than in fiscal year 1970 due to an increase of $18.7 million appropriated over the President's budget. The extent of funded and unfunded approvals for fiscal year 1971 has been projected and is summarized as follows:

Number of Grants and Percentage

	Funded		Unfunded	
Regular Program				
New	173	46%	208	54%
Renewal	152	58%	111	42%
Graduate Training				
New	- 0 -		- 0 -	
Renewal	5	42%	7	58%
Clinical Training				
New	2	67%	1	33%
Renewal	17	47%	19	53%

As in the past few years, fiscal year 1971 began with financial uncertainty and the threat of stringent appropriations. Early fiscal projections of estimated requirements against the funds available in the President's budget showed that unless negotiated 10 to 15 percent reductions in research grants were continued, no new competing awards could be made. Since the National Institutes of Health had established a 6 percent limit on research grant negotiations, a special waiver was requested by the NCI for imposition of the higher level of reduction. Because of initial difficulties in negotiating individual reductions by telephone a standard letter was devised which (a) notified the investigator well in advance of the reduction, and (b) requested that his continuation application be submitted at the reduced level. This approach proved successful and resulted in timely awards.

The NCI appropriation was finally apportioned in March and the additional money made it possible to fund all research grant awards at recommended levels. Consequently, approximately 500 grants awarded at negotiated reductions have since been or will be restored to recommended levels. In addition, the NCI will fund approximately 50 percent of the approved traditional renewal applications and 30 percent of the new applications.

While training grant funds were not reduced for fiscal year 1971, the training area did not receive the benefit of additional funds. As the summary indicates, there were no new graduate training awards. As a result of uncertainties regarding the future of training support at NIH, the NCI has not been able to make suitable program projections except in connection with the potential increases in funds to be made available under the conquest of cancer program.

Yarborough Commission Report

During the past year a national panel of consultants, composed of 13 eminent laymen and 13 eminent scientists, was established to assist the Committee on Labor and Welfare to "examine, investigate, and make a complete study of any and all matters pertaining to (1) the present status and extent of scientific research conducted by governmental and nongovernmental agencies to ascertain the causes and develop means for the treatment, cure and elimination of cancer, (2) the prospect for success in such endeavors, and (3) means and measures necessary or desirable to facilitate success in such endeavors at the earliest

possible time." After months of intensive and diligent effort the panel prepared a report, "A National Program for the Conquest of Cancer," dedicated to the proposition that the conquest of cancer should be a national crusade and recommending that a National Cancer Authority be established.

Presidential Message

The President, in his State of the Union message, stated that he was making the conquest of cancer a national goal and proposed, initially, an additional $100 million over and above the present NCI budget.

As a result of these developments the NCI staff has prepared several "program projections" in anticipation of significantly increased funding for cancer research. An important area for expansion is that of the Cancer Research Center. Existing ones should be expanded and new ones need to be developed. The Center concept involves the establishment within a medical community of a single comprehensive and multidisciplinary focus of cancer activity so that laboratory research activities can be translated quickly into clinical techniques that would eventually be utilized broadly by practicing physicians. Ideally, the Center would also serve as a focal point for continuing education in the area of cancer diagnosis and treatment.

Policy on Research Career Development Awards

Research career development awards, supported by the NIH, will henceforth be limited to a single support period of five years. The present practice of renewing these awards will be discontinued and no renewals of presently active awards will be made if the expiration date of the award is after June 30, 1972.

At the March 1971 Council meeting, the members expressed dismay and regret at this change in policy and passed a resolution requesting reconsideration by the Director, NIH, since, in the face of the decision of the President to make the conquest of cancer a national goal, and in the planning for the implementation of such a program, the acute shortage of highly trained personnel is the most prominent limiting factor. The Council added further that it is not realistic to expect the universities in their present fiscal plight to assume the obligations that the Career Development Program has supported.

Highlights of the National Advisory Cancer Council Meetings

The Council reviewed the guidelines for NCI staff in negotiating desirable adjustments in grant amounts and terms and endorsed the recommendation of staff that the item pertaining to the Change of Grantee Institution be modified to conform with recently established NIH procedure. The new procedure removes the one year limitation of support for a grantee who moves to another institution and allows support for the remainder of the project period.

At its November 1970 meeting, the Council endorsed recommendations by its Subcommittee on Diagnosis and Treatment on procedures and opportunities that could be exploited to further improve the diagnosis and treatment of cancer. Of the recommendations endorsed it was proposed that the need for rapid exploitation of the carcino-embryonic antigen (CEA) test for large bowel

neoplasms and the formation of a cooperative group to develop and evaluate diagnostic tests for cancer be explored for technical feasibility and probability of funding within the limits of our current budget programs.

It was further proposed that efforts to educate the public in the value of early diagnosis and treatment of cancer be carried out within the limits of NCI public information authority as defined by current policy.

At its March 1971 meeting, the Council recommended that immediate steps be taken to implement a coordinated national program of research and development on colorectal cancer, including the appointment of an ad hoc Advisory Committee. Preliminary plans had been approved at an earlier meeting.

The Subcommittee on Carcinogenesis and Prevention, as well as the Subcommittee on Diagnosis and Treatment, in keeping with prior Council recommendations, reviewed special actions which required special consideration before being brought to Council for final deliberation and action.

Evaluation of Cancer Centers Outside the Continental United States

During the past year, the Associate Director and the Deputy Associate Director for Extramural Activities visited cancer centers in the major cities of some of the Canadian Provinces to see how they provide service and incorporate research aspects into the delivery of cancer care. It was their opinion that the centralized cancer care facilities which have been developed in some of the Canadian Provinces, coupled with the well developed referral system, have much to offer.

Last fall, the Associate Director of the Extramural Activities area visited major components of the European Organization for Research on the Therapy of Cancer. His comprehensive travels included Brussels, Paris, Zurich, parts of Germany, Milan, and London. A detailed report was issued to members of the NACC.

PROGRAM ANALYSIS AND REPORTING SECTION

Any organization engaged in supporting medical research today needs a dependable source of accurate up-to-date information. The general area of medical knowledge has recently been expanding at a rapid rate. The requests and requirements for reliable data concerning cancer research have paralleled this expansion. The Extramural Activities Area, unlike other NCI groups, is involved in the entire spectrum of cancer research and training; ranging from the most basic studies of life processes (molecular biology), through studies of cancer in experimental animals (viral etiology), to the patient in the sterile hospital room, who is on a Phase III drug protocol study. The prime function of the Program Analysis and Reporting Section (PARS) is to supply dependable and up-to-date research information to the Associate Director for Extramural Activities and his entire staff. In addition to serving the Extramural Area, PARS also functions as a major source of information for the entire NCI. Other groups which frequently request data include the NCI Information Office and Budget Office, the Office of the Director and the Program Planning and Analysis group.

The Section compiles, analyzes and reports scientific and certain administrative data on all the extramural programs, which include research grants, training grants, career awards and fellowships. In addition to the information on extramural programs, PARS also maintains data on the contracts of the NCI collaborative areas: Chemotherapy, Etiology and General Labs and Clinics. Data are maintained in such a manner that it is possible to retrieve by five axes: major program interest area, disciplinary approach, methodology employed, disease site and the study subject (including clinical emphasis). It is possible to use any one of these axes separately or any combination of the five, to obtain the desired data. Key words and phrases are also used to assist in the selection process, for example a specific carcinogen (nitrosamine) or a drug (bleomycin).

Perhaps the most factual scientific information associated with the different extramural programs is found in the published papers of grantees. Knowledge gained from extramural support is a vital tool in the evaluation of ongoing grants and programs and is necessary for realistic planning of future activities. In order to keep Extramural Program Directors current with the published work, PARS staff prepares monthly a listing of papers published in each program area. Selected papers are also written up in condensed form. The Section is presently contemplating discontinuing the across-the-board abstracting of papers in favor of more detailed in-depth reports on specific subjects. The subjects would be selected in consultation with the Program

Directors and others, or as the state-of-the-art would indicate. It is hoped that these reports would present data which are extremely current, on the leading edge of the knowledge frontier, and could be used in the formulation of program direction and goals.

PARS, over the past few years, has built up good liaison with the staff of the American Cancer Society (ACS). Because of this, both the NCI and ACS have benefitted from the free exchange of information on both a formal and informal basis. Similar activities have been conducted with intramural staff of Chemotherapy, Etiology and the Research Information Branch of NCI.

At the request of the Cancer Council, the Extramural Activities area of NCI has been asked to develop an ongoing survey of persons who have received training under either a Graduate Training Grant or a Fellowship. The purpose of the survey is to provide data that would assist in the evaluation of the training programs. NIH is presently in the initial stages of a survey of all past trainees and fellows. It is planned to conduct a great deal of the data collection and cross-referencing by outside contract. In the past, similar follow-up studies have been conducted by actually contacting the trainees and fellows (questionnaire type study). This survey is proposed to be based largely on such sources as the National Register of Scientific and Technical Personnel (NSF) and AMA data banks. PARS, along with the NCI Training Officer, plans to work closely with NIH, OADPPE, OPA, in the survey of past trainees and fellows; and then, possibly as an extension of the NIH contract, develop a continuing ongoing operation which would update the data year by year.

The Section maintains a unique file containing documents which are useful in judging the competence and productivity of individual investigators who have applied for NCI support. This file is arranged alphabetically and all relevant information for each person is in a single folder, even though that person may have applied for several grants or awards. Inactive files were reviewed and pertinent documents copied so that the present "Investigator File" hopefully contains information on every person who has ever applied for extramural support. This file has proved extremely useful in documenting events which have occurred in the past since the main (numerical) grant files are sent to Federal Storage after having been inactive for five years. As an auxiliary to the Investigator File, there is also an "Investigator Card." Whereas the Files are prepared for Principal Investigators only, the cards are presently being up-dated to include all the professional staff associated with a grant. In the past few years the NCI has been awarding more larger grants (for example, the SKI and PO2 type grants). There are over three hundred staff members associated with the SKI grant. Such notable investigators as Howard Temin and David Baltimore, while presently not principal investigators on research grants, are being supported

by multidisciplinary NCI grants. We feel that this is an important task and necessary in order to have better information coverage. Many inquiries come to this Section regarding NCI support for specific individuals; without such a data bank it would be a lengthy task, if it could be done at all, to provide the correct answers.

PARS continues to serve as a central screening point to detect possible conflict and/or overlap between grants and contracts. The purpose of this operation is to alert responsible program people (program directors or project officers) to these trouble spots, either in workscopes and/or budgets. In many cases the difficulty lies mainly in poor documentation and not in outright attempts to obtain dual support. At institutions where both grant and contract money is awarded (sometimes to the same investigator) greater care should be taken to delineate the research proposals. Often the scientific workscopes of these contracts and grants are so intricately interwoven they appear to be a double stranded DNA molecule.

In the past, the main grant file was the official repository for all first copy reprints of published papers submitted by grantees. PARS received the remaining copies; PARS kept one and passed the rest on to other persons. As of now, the PARS reprint file will become the official file. We will keep all first copies and pass the rest on as before. Program Directors will request copies by grant number.

The appendix I which appears at the end of the Extramural Activities Annual Report is prepared by the staff of the Program Analysis and Reporting Section. Each year this report requires many man hours of staff time. Difficult as the task is, it is well worth doing, for it is the only time in the year when you can stand back and try to get a good perspective on the current status of the extramural literature.

It is obvious from the above review of some of the activities and operations conducted by PARS, that this group serves as a major source of information for all NCI programs. Moreover, because of the familiarity of the staff with the progress, problems and current status of cancer research and related fields, the Section is frequently called upon to provide information regardless of the source of support. These requests come either directly from NIH laboratory scientists or are referred to us by others.

AWARDS REVIEW AND TECHNICAL ADMINISTRATION BRANCH

The Awards Review and Technical Administration Branch provides scientific review and technical administration for both research grants and training grants assigned to the following cancer programs:

Carcinogenesis
Cancer Biology
Cancer Epidemiology
Cancer Pharmacology

In the program descriptions which follow, the number of grants includes all grants that were active as of March 1, 1971--whether funded from FY 70 or FY 71 funds. Each dollar value shown (expressed in millions of dollars) represents the total cost (direct plus indirect costs) of the grants concerned and includes FY 71 funds already obligated, plus FY 71 funds still to be obligated. In estimating the latter currently unobligated funds, the formula employed was direct costs authorized x 1.18. The factor 1.18 reflects an average 10% downward negotiation of direct costs authorized, plus a 31% indirect cost component. Grants to be awarded as a result of the applications approved at the March 1971 meeting of the National Advisory Cancer Council are not reflected in the number of grants or in the dollar values shown.

CARCINOGENESIS PROGRAM

The Carcinogenesis Program is concerned with the achievement of a more effective prevention of cancer in man, caused or promoted by chemical or physical agents, or the possible interaction of chemical carcinogens with viruses. The program seeks to encourage studies on the identification and inhibition of the preneoplastic and early neoplastic states, the in vivo inactivation of chemical carcinogens, and the development of in vitro procedures and systems for the study of the carcinogenesis process and the evaluation of suspect carcinogens. The program also supports a large amount of research on the biochemistry of host-tumor relationships and an analogous body of studies on basic biochemical research of potential significance to cancer. The several categories of research which constitute this program are further characterized in the outline shown below.

CARCINOGENESIS PROGRAM
(239 grants: $14.6 million)

I. Chemical Carcinogenesis (63 grants; $7.8 million)

1. Isolation, identification and synthesis of known and suspect chemical carcinogens and their metabolites; molecular structure - carcinogenicity relationships
2. Action, mechanism of action, and physiological disposition of chemical carcinogens: Biochemical changes in physiological compounds and processes produced by chemical carcinogens. Cytologic and histologic changes produced by chemical carcinogens; effects on cell ultrastructure and function. Properties of cells transformed by chemical carcinogens; development of carcinogenicity screening procedures. Carcinogenicity screening. Metabolism, toxicity, and excretion of chemical carcinogens and their metabolites; identification of proximate carcinogens. Factors which initiate, promote or inhibit the action of chemical carcinogens; influence of age, sex, species, and strain.

The six Program Projects and one Research Center assigned to the Carcinogenesis Program, representing an FY 71 expenditure (total costs) of approximately $5.8 million, are included in this category (I).

II. Biochemistry of Cancer and Cancerous Hosts (85 grants; $3.4 million)

1. Carbohydrates, Energy Metabolism, and Respiration of Cancer Cells, and Cancerous Hosts
2. Amino Acids, Proteins, and Ribosomes in Cancer
3. Lipids in Cancer
4. Nucleic Acids in Cancer
5. Endocrine-Related Biochemistry of Cancer and Cancerous Hosts

6. Enzymes in Cancer
7. Metabolic Patterns in Pre and Early Neoplasia
8. Development of Biochemical Methods for the Early Detection of Cancer
9. Biochemistry of Melanin in Melanoma and in Other Systems

III. Basic Biochemistry of Potential Significance to Cancer (91 grants; $3.4 million)

1. Biochemistry and Biophysics of Amino Acids and Proteins, Biosynthesis of Proteins
2. Biochemistry and Biophysics of Nucleic Acids and Related Compounds
3. Endocrinology with Potential Applications to Cancer
4. Enzymes: structure; synthesis; regulatory properties; mechanism of action
5. Other Biochemistry with Potential Applications to Cancer

In addition to research grants the Carcinogenesis Program supports ten graduate training (T01) grants, representing a total FY 71 cost of approximately $0.9 million. The training provided encompasses all three of the research categories described above.

During FY 71, as in the case of FY 70, programming efforts were severely depressed by budgetary restrictions and by uncertainties over the level of funding. In this regard the hoped for programming of interdisciplinary studies on possible interactions between chemical carcinogens and oncogenic viruses, alluded to in the FY 70 Annual Report, was not realized. Analogously, there was no increase in the number of research projects concerned with the development of *in vitro* carcinogenesis test systems, also mentioned in the latter report.

Program Highlights

Dr. Har G. Khorana (CA 11981) announced last summer that he had achieved the complete laboratory synthesis of a gene. This accomplishment marks a further fruition of his distinguished studies on polynucleotide synthesis for which he was awarded the Nobel Prize in 1968.

Dr. Howard M. Temin (CA 07175) and Dr. Sol Spiegelman (CA 02332) have been very much in the forefront of the rapidly expanding research on RNA-dependent DNA polymerase. The existence of an RNA-dependent DNA polymerase, independently discovered by Dr. Temin and Dr. David Baltimore, is consistent with the suggestion made approximately six years ago by Dr. Temin, to the effect that RNA may serve as the template for DNA. Spiegelman quickly confirmed Temin's basic findings. Moreover, Spiegelman's emergent data indicate the conversion of the Temin RNA:DNA hybrid to double-stranded DNA, a circumstance which would be expected to favor entry of the viral genetic material into the chromosomes of the host cell, with associated focus of attention on the question of possible transmission of viral genetic substance to host cell progeny.

As pertains to the mechanism of action of carcinogenic aryl polycyclic hydrocarbons, the continued efforts of some of our grantees and their collaborators have achieved understandings judged to be of prime significance. These are described below:

The work of Drs. Sukdeb Mondal and Charles Heidelberger (CA 07175) on the exposure of single cells to a carcinogenic hydrocarbon and the subsequent study of the progeny of the single cells, clarifies the role of agent toxicity in the transformation process and lends strong support to the view that the carcinogen does not select for preexisting malignant cells. It appears that the toxicity of the carcinogenic hydrocarbon is not directly related to its effectiveness in producing cell transformation, since the frequency of transformation was less than 100 percent at carcinogen concentrations both lower and higher than optimal, whereas toxicity (decreased cloning efficiency) appeared only at carcinogen concentrations higher than those producing 100 percent transformation frequency of the clones derived from single cells which had been exposed to the carcinogen. The fact that carcinogen-treated single cells produce clones all of which contain transformed colonies, argues strongly in favor of the view that the carcinogenic hydrocarbon converts nonmalignant control cells to malignant cells. It remains to be determined whether this apparently direct transformation is achieved by the chemical carcinogen acting alone or in association with an oncogenic virus.

Dr. Leila Diamond's research (CA 08936) on the metabolism of 7,12 dimethylbenz(a)anthracene (DMBA) to water-soluble derivatives has focussed on a possible mechanism by which DMBA becomes activated to its carcinogenic form. Dr. Diamond has initially shown that DMBA is metabolized to water-soluble derivatives primarily by those cells (normal rodent cells and Hela cells, in contrast to transformed rodent cells and human diploid cells) in which cell multiplication is inhibited by DMBA. In addition, Drs. Diamond and H. Fred Clark have more recently found that many poikilothermic vertebrate cell cultures are able to metabolize Benzo(a)pyrene (BP) and that cell multiplication is inhibited by BP only in those cultures in which the metabolism of BP is highly efficient. The cooperative studies of Drs. Diamond and Harry V. Gelboin reveal that inhibition of the capability of embryo hamster cells to metabolize DMBA is associated with resistance of these cells to the cytotoxic effects of DMBA. In the latter studies, inhibition of DMBA metabolism was achieved by pretreatment of the cells with 7,8 benzoflavone (BF) which also inhibits the aryl hydrocarbon hydroxylase activity in homogenates of induced hamster embryo cells. The more recent data of Drs. Gelboin, Friedrich Weibel and Diamond would appear to suggest that the tumorigenesis inhibitory activity of 7,8 benzoflavone when this compound is applied topically to mouse skin together with DMBA, is due to inhibition of the aryl hydrocarbon hydroxylase and that the activity of the latter may have a role in the conversion of DMBA to its carcinogenic form.

CANCER BIOLOGY PROGRAM

The Cancer Biology program is designed to provide fundamental information on the causes and nature of cancer in man, with the expectation that this will result in better methods of diagnosis, prevention and treatment of neoplastic diseases. The program comprises the areas of Viral Oncology, Tumor Biology and Cancer-related Biology.

Viral Oncology is concerned with the role of viruses in the etiology of cancer and the consequences of this for the identification of susceptible individuals, early diagnosis, and development of new modes of prevention and treatment. Investigations include studies at the cellular and molecular levels as well as the animal level.

In Tumor Biology the emphasis is on the preneoplastic and neoplastic lesion itself, on the tumor-host relationship in its various manifestations, and on the biology of the tumor cell.

Cancer-related Biology encompasses aspects of cell biology, molecular biology, embryology, genetics, morphogenesis, pathology and other disciplines that may range beyond the confines of manifest cancer research, but contribute significantly to it, conceptually and experimentally. While the greatest interest resides in the biology of animal cell systems, projects which involve other forms of life, if these offer particular advantages, are also considered to be appropriate for the cancer biology program.

The program, as it prevailed on March 1, 1971, consisted of the following: 185 regular projects, $8.4 million; 7 program projects, $1.8 million; and 19 training grants, $1.2 million; a total of 204 grants and $11.4 million, thus exhibiting a sharp drop from fiscal year 1970, during which, at a comparable time, there were 251 grants at a cost of $13.6 million.

Restoration of administrative reductions and activation of more approved projects as a result of additional funds provided the National Cancer Institute in fiscal year 1971 will restore the program to approximately 255 grants and $14.1 million by the end of the fiscal year, viz., 229 regular projects, $10.9 million; 7 program projects, $2 million; and 19 training grants, $1.2 million. With regard to research and program projects, the allocation would be as follows: Viral Oncology, 107 grants, $7.1 million; Tumor Biology, 51 grants, $3.8 million, and Cancer-related Biology, 78 grants, $2.0 million.

Significant Accomplishments and Trends

RNA tumor viruses are possibly the most common of the viral agents responsible for spontaneous neoplastic disease, and also the most complex. Progress in this segment of the Cancer Biology program has taken a leap forward after trailing behind the accomplishments in the DNA tumor virus area. Until now it was a puzzle how a gene of a tumor virus of the RNA type could stably attach to cellular genes, since these consist of DNA. NIH grant-supported discovery of an enzyme in the RNA tumor viruses, an RNA-dependent DNA polymerase ("reverse transcriptase"), which synthesizes complementary DNA, the first enzyme of this type known, provides a potential answer to this puzzle, and, at the same time

opens up new avenues for etiological and epidemiological studies of cancer in man. Much remains to clarify the function and specificity of this enzyme in viral oncogenesis.

Among other notable achievements has been the demonstration, by means of a mutant of Rous sarcoma virus, that the continued expression of a viral gene is needed for maintenance of transformation, implying that such a gene is indeed integrated into the genome of the host, presumably through the action of the transcriptase. Evidence has also been adduced that the congenital transmission of the avian leukosis virus, also of the RNA type, is accomplished in the form of DNA, consistent with the role of the newly discovered enzyme. Finally, a significant distinction between the constitution of the RNA of transforming and non-transforming variants of an RNA avian tumor virus has been reported.

Progress in two outstanding problems in oncogenesis - the mechanism of viral transformation and its primary expression - has been significantly advanced by the continued exploitation of mutant DNA tumor viruses. Of particular interest is the increasing evidence pointing to a change at or near the cell periphery as the crucial and possibly primary expression of virus-controlled oncogenic transformation. Thus, a characteristic surface change in hamster cells transformed by a temperature-sensitive polyoma mutant capable of replicating in appropriate cells at one temperature but not a higher one, was itself temperature sensitive; that is, it exhibited the change at one temperature but not the other, thus closely relating the transformation function of a viral gene with its phenotypic expression. Induction of host DNA synthesis under otherwise inhibitory conditions, another characteristic property of these viruses, was shown to be similarly controlled. On the other hand, certain other attributes of transformed cells, such as growth in agar, were not temperature controlled, and may therefore represent only secondary changes.

A surface change similar to that which occurs in oncogenic transformation was shown to occur temporarily in normal cells during mitosis or upon mild exposure to proteases. There is evidence that the change results from transient exposure of otherwise hidden sites at or near the surface of the cell, and which may be a factor in the control of cell division. In virus transformed cells the exposed sites become fixed.

Disregarding the question of whether viruses are a significant factor in human cancer although, of course, efforts in this endeavor are continuing unabated, research in basic viral oncology is thought likely to bring us closer than any other line of inquiry to a precise understanding of what makes a cell malignant. Furthermore, in addition to studies on viruses as infectious and transforming agents, these agents may function and, indeed, be potentially useful as carriers of genetic information from one cell or host to another, providing a powerful tool in the analysis of growth regulation of animal cells, in probing gene expression in molecular terms, and in possible intervention in genetic diseases.

In the Tumor Biology area, one of the most intriguing developments is the possibility of reversing the neoplastic behaviour of cells. It has been claimed that certain plant tumor cells can be completely restored to normal behaviour. In animal cell systems, substances such as bromodeoxyuridine (BUdR), dimethyl sulfoxide (DMSO), and monovalent concanavalin A (CCN A), at concentrations which

do not appreciably affect normal cell growth, will reverse in vitro certain properties of transformed cells, frequently also inhibiting their malignancy, as determined by transplantation. These effects have thus far been reversible.

The relationship between differentiation and neoplasia is of interest in the above connection. It appears that those properties of a cell which have been most recently acquired are also those most readily suppressed. Thus, BUdR which reverses malignant transformation also reverses differentiated properties without seemingly affecting basic cell functions. In other instances, as with DMSO, malignancy is readily suppressed while differentiation is not affected, consistent with the idea that malignancy is the more superficially imposed of the specialized cell functions. Clearly, the mechanisms by which these repressants act differ, and much remains to learn about this. These observations also suggest a modified approach to chemotherapy; the goal would be to repress malignant behaviour of cells rather than destroy them. In vitro systems are admirably suited for intensive screening efforts to isolate or synthesize new types of reversants.

In the Cancer-related Biology segment of the program, research in somatic cell hybridization continues to expand and gene mapping of mammalian cells is progressing rapidly. This technique is of theoretical and practical interest in virology, oncology, cell physiology, molecular biology, and of potential value in the treatment of genetic diseases.

Future Goals

Because of its central position in neoplasia, and because of the encouraging state of the art, Cell Biology is proposed as the focus of expanded effort in the Cancer Biology program. The following areas are identified for particular emphasis:

1. The precise mechanism of malignant transformation by tumor viruses, both of the DNA and RNA type, and identification of the primary phenotypic expression of transformation.

2. Reversal of neoplastic behaviour of cells.

3. Cellular regulatory mechanisms, particularly the mechanism of cell division, and of contact inhibition of replication.

4. Cell hybridization and its applications.

5. Improved methods to isolate in pure culture various mammalian cells, including tumor cells. Better methods are needed to identify, characterize and maintain cells in a stable state, and for mass culture of lymphocytes for possible therapy, of neoplastic cells for the preparation of specific antigens, and of specialized cells for specific products.

Expansion of certain existing programs and creation of new ones to carry out these additional efforts have been considered and tentatively identified.

Administration

For more effective operation of the Cancer Biology program, particularly appropriate in this time of expected expansion, the creation of a team consisting of the Program Director, a Grants Assistant to administer the day to day operation and more routine aspects of the program, a Grants Manager to control fiscal matters, and two secretaries, should be considered. Such an arrangement would provide the program director further opportunity to develop the substantive components of the program.

CANCER EPIDEMIOLOGY

The extramural grants program in cancer epidemiology encompasses two main subprograms, epidemiology and biometry, and two minor subprograms, one in the behavioral sciences and the other, for administrative purposes, in biomedical communications.

Representative research in cancer epidemiology aims traditionally to signal the urgency of a cancer problem and to generate hypotheses about the causes or the course of cancer for systematic controlled investigation in the laboratory or clinic. Of more importance, it seeks to verify the generality in human populations of hypotheses evolving from the laboratory and the clinic. The epidemiologist finds and analyzes the frequency distributions of cancer of different forms, processes and stages in selected sites of the body. He particularly studies how changes in these distributions relate to different forms, degrees and interactions of personal and environmental characteristics. Examples of personal characteristics include other pathologic states and physiologic, familial, ethnic, occupational and psychologic factors. Examples of environmental characteristics include geographic, atmospheric, chemical, viral, economic and social factors.

Representative research in cancer biometry aims to contribute to the design of convincing investigations not only in epidemiology but also in the laboratory and the clinic. Involved are (1) the measurement of the different forms, processes and stages of cancer at different sites of the body, (2) the measurement of the personal and environmental characteristics that may bear antecedent, concurrent or consequent relationships to the various forms and processes of cancer, (3) the modification of existing mathematical and statistical models and the invention of new ones for the prediction of the course of cancer and for the convincing design and analysis of research on cancer problems, and (4) the application of computer technology to the design, conduct and analysis of cancer research.

During fiscal year 1971, 22 projects were active at a cost of 1.4 million dollars--1 project less than in the previous fiscal year but $68,000 more. An increase of $6,000 in average cost per grant seems to reflect no significant change in pattern of support other than can be attributed to minor expansion of activities and the general rise in the cost of research. Although during FY 70 half the support came from previous fiscal years' funds, and half from the current, in FY 71, 85% of the support came from previous fiscal years' funds and only 15% from the current. This change may reflect the reduction in the availability of grant funds during the current fiscal year. During FY 70, 89% of the support went to research, and 11% to training; in FY 71, 87% to research and 13% to training. During FY 70, 55% of the support went to epidemiology, 29% to biometry, and 16% to biomedical communications; in FY 71, 58% to epidemiology, 32% to biometry, and 10% to biomedical communications. Thus, this year has brought no significant change in the pattern of support. (The termination of one of the two grants in biomedical communications accounts for the decrease in that area.)

A change in the pattern of support does appear, however, in the targets of research in the subprogram of cancer epidemiology. Although most of this support is in the form of research program-project grants (twice as many dollars as in research projects), and although research program-projects have each a number of targets, estimates have been arrived at for the amounts of support going to separate targets in both research program-projects and research projects. The percentage of these funds going to cancer of the breast has remained constant from FY 70 (26%) to FY 71 (25%) as have the funds to environmental chemical carcinogenesis (10% in both years); the percentage to sociologic variables in the prevention of cancer has decreased slightly (19% in FY 70, 16% in FY 71). The major changes have been in cancer of the cervix, up from 21% in FY 70 to 32% in FY 71, and in cancer of the stomach, down from 20% in FY 70 to 7% in FY 71. The support to cancer of the cervix increased by 84 thousand dollars, and the support to cancer of the stomach decreased by 71 thousand dollars.

The increasing emphasis on cancer of the cervix is encouraging, but the decreasing emphasis on cancer of the stomach should be remedied, for knowledge of the epidemiologic characteristics of cancer of the stomach is far from complete. Epidemiologic studies of cancer of other sites of the body, particularly of the colon and rectum, will need to be stimulated without, however, neglecting further work in the areas already receiving emphasis, particularly environmental chemical carcinogenesis. Other foci toward which major efforts need to be stimulated in the future include the development and utilization of precise, efficient and practical methods of assaying carcinogenic compounds for studies of large populations, the recruitment of experimental epidemiologic studies, the detection and management of the behavioral effects of cancer, the application of infectious disease epidemiology to the problems of cancer, the development and utilization of precise, efficient and practical methods for detecting indicators of cancer, the improvement of the scientific quality of requests for support of epidemiologic investigations, and the attachment of epidemiologic investigations to existing projects in chemical and viral carcinogenesis.

Some progress has begun in these efforts. An infectious disease epidemiologist has a grant in support of a research project on the viral etiology of cervical cancer, with particular emphasis on herpes virus, cytomegalovirus and trachoma inclusion conjuctivitis agents. Another investigator has a grant for a major research project on the detection of polymorphic genetic markers of cancer.

During the next fiscal year, in collaboration with the Program Analysis and Reporting Section, Extramural Activities, an attempt will be made to identify the inclusion of the epidemiology, biometry and behavioral aspects of cancer within projects supported by other NCI extramural grant programs.

The expansion of these efforts will be handicapped, however, without the production of cancer epidemiologists through training projects.

PHARMACOLOGY PROGRAM

The functions of the Pharmacology Program Area are concerned with the scientific and administrative aspects of research supported by federal funds in the preclinical and clinical phases of cancer chemotherapy. In broad terms, the workscope involves a coordinated program of research for the development and evaluation of drugs which act specifically or selectively against malignant growth with minimal toxicity to the host. The program objective is to provide necessary financial support and to encourage investigators in various scientific disciplines to pursue research for development of new approaches to more effective chemotherapeutic control of cancer. Problems relating to control and therapy are multidisciplinary in their scientific nature. These involve chemical, pharmacological and cellular aspects of abnormal as well as normal life processes. The program includes investigations, not only those directly related to malignant cells, but also some broadly based fundamental studies in biomedical sciences.

The research program currently supported by grants in this area is classified into four primary categories, each representing a successive stage in the development of new chemotherapeutic agents toward eventual clinical use. (1) Synthesis and Isolation of new chemical agents and of well-defined products from natural sources as potential anti-cancer drugs. (2) Preclinical Drug Evaluation including screening tests, experimental therapeutics, dosage regimens and preclinical pharmacologic investigations. (3) Mechanisms of Drug Action, physiological disposition, drug distribution, biotransformation and related pharmacologic aspects. (4) Clinical Pharmacology and Drug Development research in synthesis, experimental therapeutics, selective toxicity and combination therapy studies in selected clinical investigations.

During fiscal year 1971, the Pharmacology Program provided grant support to 203 individual research projects with $8.54 million. Two relatively large programs of collaborative cancer research in the preclinical and clinical areas on a broad and flexible basis involved $0.86 million. An additional $1.23 million was awarded to 50 training projects through graduate training grants, research career awards and fellowships for development of potential research investigators. The total fiscal commitment in the Pharmacology Program is $10.63 million. The available resources are distributed among the component areas of the program in proportion to their relative significance. Cognizance of scientific progress in each subcategory of the program is maintained on a continuing basis through review and analysis of periodic reports and publications of grantees. The following is a summary of the research content and fiscal apportionment within the program categories:

1. Synthesis and Isolation of Potential Anti-cancer Compounds: (106 grants; $4.03 m.) Support of projects involving synthesis of new drugs and studies on isolation of products from natural sources represents approximately 37 percent of the total funds. Twenty-two of the 106 projects are devoted to development of potential anti-cancer agents from microbial, botanical and animal sources.

Two new projects are being initiated for laboratory synthesis of rare alkaloidal compounds. Increased emphasis is being maintained on natural products research. Competent investigators are encouraged to conduct research in specific areas which remain unexplored. Eighty-four grants are involved in research in new syntheses or preparation of chemical entities. These projects include development of analogs of amino acids, sesquiterpenes, steroids, nucleic acid derivatives, proteins, organometallic compounds and specific heterocyclic ring structures.

2. Preclinical Drug Evaluation: (22 grants; $0.79 m.) A total of $0.79 m. or 8 percent of the funds committed in the Pharmacology Program are assigned to 22 research projects involving various facets of preclinical drug evaluation--development of new screening procedures, experimental therapeutic and preclinical pharmacological studies seeking new and promising leads for clinical application. This group also includes basic studies on the pharmacological and biochemical aspects of hormone therapy and experimental therapeutics of drugs administered as a single entity or in multiple combinations.

3. Mechanisms of Drug Action: (69 grants; $3.19 m.) This represents an increase of about 20 percent in the total effort devoted to mechanism of action studies. The scope of these investigations includes physiological disposition, preclinical experiments on drug distribution and biochemical studies on the mode of action of known and potential anti-cancer drugs. This category represents 30 percent of the funds available in the Pharmacology Program area. Intensive studies on drug metabolism have provided significant clues for better understanding of the mechanisms of action and for more effective clinical application of chemotherapeutic agents.

4. Drug Development and Clinical Pharmacology: (6 grants; $0.53 m.) One interdisciplinary research project supported by a grant of $0.25 m. involves a broad drug evaluation program which includes successive phases of synthesis, experimental therapeutics, physiological disposition and initial clinical trials of new drugs. In addition, there are five projects in clinical pharmacology for studies on toxicological and therapeutic aspects of new drugs in man. The funds represent approximately 5 percent of the total effort.

5. Research Program Projects: (2 grants; $0.86 m.) These awards represent support of a flexible nature at the university department level and are usually directed toward a range of problems within broad scientific disciplines to provide a central research focus rather than toward a specific single project. There are two relatively large grants to departments of pharmacology and medicine to provide personnel and special equipment facilities. Preclinical as well as clinical aspects are included in the overall investigations.

6. Research Training: (50 grants; $1.23 m.) The purpose of the training and research career fellowship awards is to encourage and to extend training of investigators preparing for research and academic careers in basic, preclinical and clinical scientific disciplines related to pharmacology. The overall distribution of support during the period was as follows: Graduate Training Grants 12; $0.68 m. Research Career Programs 11; $0.3 m. and Fellowships 27; $0.25 m. This program involves 151 trainees and fellowship awardees.

Recent Accomplishments and Promising Leads for Future Research

1. Synthesis of cephalotaxus alkaloids. The alkaloid cephalotaxine and its esters harringtonine and isoharringtonine have shown unusually high activity against P-388 leukemia with relatively low host toxicity. These are potential candidates for clinical evaluation. Because of limited availability from natural sources, development of synthetic procedures is of primary concern to the program. Dr. Steven M. Weinreb, Fordham University, (CA 12568) conducts a series of ingenious reactions and logical steps for a feasible synthesis of the alkaloid and its derivatives from readily available compounds. Dr. Frederick C. Tahk, Kent State University, (CA 12567) has devised well-conceived rational synthetic pathways for synthesis of this alkamine and conversion to natural esters as well as to other novel variants. The novel chemical approaches from Dr. Weinreb and Dr. Tahk hold excellent promise of obtaining these rare compounds in adequate amounts to pursue clinical application.

2. Selective toxicity of antitumor drugs. With his classic studies on parabiotic rats, Dr. Kurt Reissmann, University of Kansas Medical Center, (CA 08736) initiated in 1950 the concept of humoral regulation of blood cell production. His research studies on protein metabolism, uremia and effects of drugs in undifferentiated stem cells demonstrated that various cycle-dependent drugs inhibit the action of erythropoietin by different mechanisms. He will study the effect of various tumor-inhibiting drugs in effective red-cell production by Fe^{59} incorporation and myelopoiesis measurements by labeled metamyelocytes. The kinetics of granulocytopoiesis, erythropoiesis and thrombocytopoiesis will be investigated in systematic investigations to ascertain selective effects of anti-cancer drugs on the regeneration of hematopoietic tissue. The findings will contribute to knowledge for more effective clinical application of chemotherapeutic agents.

3. Development of predictive tests for clinical effectiveness. Dr. Gerald A. LePage, University of Texas M.D.Anderson Hospital and Tumor Institute, (CA 11788) demonstrated inhibition of ribonucleotide reductase with arabinosyl 6-mercaptopurine. A correlation exists between inhibition of this enzyme in cell-free systems and response of the tumor. Clinical studies will determine whether this enzyme assay can predict resistance or susceptibility to therapy. Clinical work is contemplated with the alpha- and beta-deoxythioguanosines. Extracts of mouse tumors phosphorylate both nucleosides, but bone marrow phosphorylates only the beta-anomer. Research on arabinosyladenine shows that there are wide variations in the rates at which ribosyl and arabinosyl derivatives of adenine are deaminated in different tissues. The deaminases will be studied in various tissues in an attempt to correlate the differences. Investigations on kinases of anomers will provide the basis for development of reliable predictive tests.

4. Mechanisms of drug inhibition of human leukemic cells. It has been demonstrated that tumors have abnormally high activities of one type of DNA polymerase--that is DNA polymerase preferring denatured DNA as primer. This metabolic abnormality is present in each of the two dozen human and rodent tumors and leukemias. The enzyme is specifically bound to smooth membranes

in the cytoplasm, in contrast to the DNA polymerase which prefers native DNA and which is bound to ribosomes as it occurs in the cytoplasm. Dr. John Laszlo, Duke University Medical Center, (CA 08800) will purify and characterize these enzymes and will attempt to ascribe synthetic or repair functions to them. One or more of these polymerase enzymes exert a controlling influence in DNA synthesis. Preliminary experiments in synchronized systems suggest that the polymerase enzymes are induced prior to the enhanced DNA synthesis. It appears that human chronic lymphocytic leukemia cells have a marked increase in DNA repair following exposure to UV-irradiation. This is the first significant difference in DNA metabolism between normal and chronic leukemia lymphocytes. The biochemical basis of this difference will be determined by investigating each of the enzyme steps involved in the repair of DNA injured by mechanisms such as UV-irradiation, x-ray irradiation and alkylating agents. The studies on the mechanism of action of new or established chemotherapeutic agents on human leukemic cells will continue.

5. Separation of blood elements by continuous flow centrifuge. Considerable progress is being made by Dr. Edward D. Thomas, University of Washington, (CA 10167) in technical aspects of the continuous flow blood centrifuge through adoption of the arteriovenous silastic-teflon shunts. It is possible to obtain large quantities of viable granulocytes, lymphocytes and platelets. The use of this centrifuge for plasmapheresis through exchange transfusions is proving beneficial to patients with hepatic coma. The centrifuge will provide the means for separation of large numbers of platelets for support of patients with bone marrow failure. This productive and promising research effort must continue in order to improve existing techniques and to resolve problems in the separation, preservation and infusion of platelets and other blood constituents to cancer patients.

Administration

Recent experience in dealing with scientific problems and discussions with investigators and grantees indicate need for changes in administrative aspects which seem desirable. (1) Support of research grant projects should be placed on the "forward funding" principle similar to the mechanism currently in use for training programs. Such arrangement would eliminate uncertainties regarding available funds and thus permit implementation of a planned and more effective research program. (2) For more effective coordination of financial and management aspects, it would be most desirable to have the Pharmacology Program area assigned to a single grants management specialist from the Grants Operations and Management Section.

Analytical approaches are applied to identify the scientific character of individual research projects in relation to objectives of the program. An assessment is made of the need for support in specific areas as a basis for coordination and more effective implementation of research effort. To help determine the direction of future effort and to select specific areas of research which are likely to yield the most promising clues, it is desirable to provide continuing periodic review of newly acquired knowledge. There is need for extension of support for research-trained personnel in preclinical pharmacology at the graduate and postdoctoral levels. Provision of major facilities for laboratory equipment to departments of pharmacology and to

clinical centers is considered appropriate. Cancer Research Center programs provide an excellent environment for training of clinical pharmacologists and the necessary background for research in experimental therapeutics, design, objective criteria for evaluation of therapeutic response, toxicity and ancillary studies on selective action of drugs in man.

Future Plans

Because of the inadequacy of funds for support of research, it was not possible to initiate most of the new programs projected in last year's report. These plans are being carried over to the forthcoming year. The overall direction of research effort and principal objectives of the Pharmacology Program will continue essentially as outlined. Greater emphasis will be placed in specific areas of biochemical and pharmacological research. If adequate research grant funds are available, new programs will be considered for future support. The plans are based on the need for additional emphasis on research in new areas which are not adequately represented in the current program. These are: (1) Development of Pharmacology Program Centers for collaborative research effort in preclinical and clinical pharmacology, selective toxicity, physiological disposition of drugs, mechanisms of therapeutic and pharmacologic activity. (2) Intensified research in isolation, evaluation and synthesis of natural products and laboratory syntheses of rare compounds. Extensive sources in the marine, botanical and zoological products still remain untapped. (3) Extension of present research and initiation of new projects in pharmacological and biochemical studies on specific neoplastic entities, (e.g., melanomas, neurologic tumors, adrenal tumors) for an understanding of mechanisms and development of biochemical diagnostic tests. (4) Development of systems to predict effects of chemotherapeutic agents in man by prior testing either by "in vitro" procedures or by transplantation of excised human tumors into conditioned laboratory animals. (5) Development of new instrumentation and techniques for refinement of existing procedures for isolation and identification of biotransformation products, instrumental development of platelet and blood element transfusions, prevention of infection and parabiotic procedures. (6) Research on enzymatic patterns and requirements of normal and neoplastic cells. A search for specific metabolic requirements toward rational therapeutic approaches. (7) Support through special individual grants for development of promising new investigators for an independent research career in cancer pharmacology.

CLINICAL INVESTIGATIONS BRANCH

The Clinical Investigations Branch, now in its third year, continues to be responsible for the administrative and scientific guidance and evaluation of the grant-supported cooperative clinical research of the National Cancer Institute. This Branch also administers certain closely related grant programs--Protected Environment, Leukemia Center, and selected support, liaison, and training programs.

The Cancer Clinical Investigation Review Committee provides the initial review of all grant applications for this program, and is also charged with providing the evaluation and advice on program content and direction.

During Fiscal Year 1971, the Extramural Program Directors for Radiation, Immunology, and, just toward the close of the year, Chemotherapy, have become members of the Clinical Investigations Branch. This administrative change is in the interest of increased coordination among the various therapeutically oriented programs of Extramural Activities. The reports for Fiscal Year '71 from these programs are presented separately.

COOPERATIVE CLINICAL RESEARCH PROGRAM

Changes in Fiscal Year 1971

During the year covered by this report, the increasingly thorough review procedures of the Cancer Clinical Investigation Review Committee led to the discontinuance of the Surgical Adjuvant Bladder Carcinoma Group and the Pacific Veterans Administration Cancer Chemotherapy Group. Both groups had been operating within the recent past in a quasi-probationary status in recognition of their defects.

Two new groups have been reviewed and are in the process of being funded. The Gynecologic Oncology Group is a descendant of the Surgery Adjuvant Uterine Study Group and will assume some of the long-term follow-up functions of that group. It is of considerable interest that the membership of this group includes gynecologists, radiation therapists, and chemotherapists, all of equal status as co-investigators.

The Radiation Therapy Oncology Group, developed in part under the auspices of the Committee for Radiation Therapy Studies, is composed primarily of radiation therapists, but their protocols will require multidisciplinary cooperation in each participating institution. It is expected that several, if not all, of the ongoing single-protocol radiotherapy groups will merge in time into the Radiation Therapy Oncology Group as the members of the RTOG include many of the members of these other groups.

The Surgical Adjuvant Breast Group has been reorganized extensively and funded to conduct a long-overdue prospective controlled comparison of radical

mastectomy versus lesser procedures for primary carcinoma of the breast. This group now consists of surgeons and radiation therapists, again of necessity co-equal partners in the endeavor.

At the direction of the National Advisory Cancer Council, the Lymphoma Task Force and the Chronic Leukemia Task Force have been discontinued. These Task Forces had been supported through the Clinical Investigations Branch and the results of their deliberations were of value to the Program, but their role has been taken over in other fashions which are expected to be more effective. The Breast Cancer Task Force continues actively, and increased clarification of the interrelationships between the breast cancer programs of the Clinical Investigations Branch and the Task Force is under way.

The currently active grant-supported cooperative groups are the following:

Chemotherapy

Acute Leukemia Cooperative Group B
Central Oncology Group
Children's Cancer Study Group A
Cooperative Breast Cancer Group
Eastern Cooperative Oncology Group
Polycythemia Vera Study Group
Southeastern Cancer Chemotherapy Study Group
Southwest Cancer Chemotherapy Group
Western Cooperative Cancer Chemotherapy Group
Wilms' Tumor Study Group

Surgery

Gynecologic Oncology Group
Surgical Adjuvant Breast Group
Surgical Adjuvant Renal Cell Carcinoma Group
Veterans Administration Urological Research Group

Radiotherapy

Preoperative Irradiation Lung Cancer Group
Radiotherapy-Chemotherapy Head and Neck Cancer Group
Radiotherapy Hodgkin's Disease Group
Radiotherapy Prostatic Cancer Group
Radiation Therapy Oncology Group.

The total support of this program for Fiscal Year 1971 is $7.4 million.

There are 1241 investigators at almost 300 institutions throughout the country who participate in this program. Approximately 230 active protocol studies are under way by these investigators and new protocols are being implemented at a rate of approximately one a week.

Seminars and Symposia

With the guidance and assistance of the Cancer Clinical Investigation Review Committee, the series of symposia on topics identified as of major interest and importance by the members and chairmen of the groups continues to be useful and productive. The third symposium in this series was a conference on Tumor Immunology held November 9-10, 1970, in Atlanta, Georgia. The proceedings have been transcribed and will be distributed to all members of the Cooperative Group Program. This symposium evoked considerable interest and enthusiasm. Requests for copies of the proceedings have come from many other interested persons not currently participating in the Cooperative Research Program.

The fourth symposium was on Statistical Aspects of Protocol Design and was held in San Juan, Puerto Rico, December 9-10, 1970, in conjunction with the American Society of Hematology meetings. The emphasis at this meeting was to develop the communication and working relationship between the group biostatisticians and other members of the cooperative groups, and to have presented some newer approaches toward biostatistical evaluation of cooperative group studies. The proceedings of this well attended conference have also been transcribed and will be distributed in a fashion similar to that to be utilized for the Immunology Conference.

The fifth meeting of this series was a Workshop-Symposium on Cell Kinetics, Cell Biology, and Solid Tumor Therapy held at Brook Lodge in Augusta, Michigan, March 22-24, 1971. This was a working meeting, participants of which included the CCIRC, selected scientists working in the various fields included in the material to be covered, and investigatively oriented clinicians who all jointly participated in development and exploration of possibilities for new drugs in solid tumor therapy. Three concurrent workshops were held, one dealing with colorectal cancer, one concerned with lung cancer, and one regarding bladder cancer. The ideas and deliberations evolving from this meeting will be collated into a document setting forth the conclusions of the workshop. This will be made available to cooperative group membership and to such others as are interested.

A clinically oriented symposium on the state of the art in diagnosis and treatment of Hodgkin's Disease is planned for October 7-9 in St. Louis, Missouri. This meeting will be open to the medical public as well as to the cooperative group membership, and proceedings are planned to be published in the Archives of Internal Medicine. This meeting is being held in conjunction with the membership of the former Lymphoma Task Force, and is primarily an educational endeavor.

The next symposium currently under development is a working conference on criteria of response in solid tumors. It will be held in mid-Fiscal Year 1972.

Direction for the Future

It is becoming increasingly apparent that there is considerable interdisciplinary overlap with respect to the studies designed by the various groups,

and the membership of the groups themselves. This is a healthy development as it provides for more sophisticated studies and involves more individuals in controlled clinical investigations of cancer therapy of all sorts. Many of the groups are planning, or have under way, studies in which the two groups jointly utilize a common protocol. The cross fertilization expected to result as a consequence of the organization of the Radiation Therapy Oncology Group will continue to supplement this process.

Future study of unusual pediatric solid tumors, the program of intergroup liaison working parties developing single protocols to be utilized by pediatricians of three chemotherapy groups, is progressing satisfactorily. Patient entry on new protocols for the evaluation of therapy of Ewing's tumors and Rhabdomyosarcoma is expected to begin during Fiscal Year 1972.

As a result of some of the former symposia jointly held by the Clinical Investigations Branch and the Cancer Clinical Investigation Review Committee, and of the series of 'mini-symposia' held in connection with the CCIRC meetings, new guidelines are being developed with respect to various areas of the program. The continued development of these guidelines is serving a dual purpose in that it provides clearer understanding for the cooperative membership grant applicants with respect to their responsibilities to the program, and it provides more uniform materials from the grantees for the review and evaluation processes of the Branch and the CCIRC. This trend will continue in the ensuing year.

The trans-Atlantic liaison and cooperation with the European Organization for Research in the Treatment of Cancer has increased during FY-71 in part as a consequence of National Cancer Institute and CCIRC representation at EORTC meetings, and attendance at CIB and CCIRC symposia by members of the EORTC. Informational material regarding the results of protocol studies being carried out by the American and European groups is being exchanged regularly. The grant support provided the central organization of the EORTC through the CIB has been increased, and it is expected that this will materially aid the expansion of the EORTC activities to countries in Europe not currently represented in the EORTC. Exploration of further means of increasing communication and cooperation between these two large communities of cooperative clinical cancer research is continuing within the constraints imposed by limitation of funding on both sides.

It is the concern of the Clinical Investigations Branch, the CCIRC, and the various cooperative group chairmen that the medical community at large has insufficient awareness of both the multitude and the importance of the prospective randomized therapeutic studies being carried out in this program. Among the efforts under way to increase this awareness and to promulgate the greater acceptance of the concept of controlled therapeutic testing, an annotated bibliography of the publications of the various components of the cooperative clinical research program is being developed for wide circulation. This document should be of considerable value both to people interested in bibliographies concerning cancer therapy and as an example of the importance of assessing therapies in this fashion.

As awareness of the importance of adequate data processing and statistical support for cooperative groups has developed, the CCIRC and the CIB jointly have reviewed this entire aspect of the program through the Ad Hoc Coordinating Center Study Group. A highly respected private statistical consultant has also reviewed the data processing and biostatistical operations of the cooperative groups and his report has been an important document for aiding the deliberations of the CCSG. The recommendations of this group have been presented to the CCIRC and it is expected that emphasis will be placed on seeking increased fiscal support for this particular aspect of the program in the coming year.

LEUKEMIA CENTER (PLATELET) PROGRAM

During the past five years, funds have been available through a special appropriation for providing supportive care for the management of thrombocytopenia as a consequence of intensive chemotherapy of leukemia. The results of this effort to date have confirmed that massive platelet transfusions do indeed reduce the mortality rate from hemorrhage in acute leukemics significantly.

Following a symposium on platelet transfusion held in April 1970, the CCIRC and the CIB reviewed the current status of platelet transfusions. It was concluded that platelet transfusion for thrombocytopenia was no longer a research problem but was current practice and no longer required support from research funds. A new set of guidelines for the Leukemia Center (Platelet) Program was developed, circulated to the grantees under this program, and the majority of the grant applications for support from this program have been revised and reviewed in the light of the new guidelines. The sophistication, extent, and potential yield in platelet research that seems likely from this new direction of the program appear most promising indeed.

This program currently has 28 grantees, and the fiscal support for it is $1.5 million per year.

PROTECTED ENVIRONMENT PROGRAM

Beginning in Fiscal Year 1966, grant support has been provided for the study of intensive reverse-isolation techniques designed to prevent serious infections in patients undergoing vigorous chemotherapy for neoplastic disease. These techniques have involved the use of either the Life Island or the Laminar Air Flow units in which everything coming in contact with the patient has previously been sterilized. These units require major personnel support of all categories of professional and para-medical personnel and also require extensive bacteriologic monitoring.

As a consequence of a two-day conference held in FY-70 and another updating symposium held September 21, 1970, a new set of guidelines for this program has been developed and supplied to the grantees. Review of the grant applications has been conducted in the light of these guidelines. Inasmuch as two of the participating institutions have developed serious local problems which interfered with their conduct of the studies, it is likely that the

size of this program will decrease in FY-72. Efforts are under way to seek institutions capable of and interested in participating in this program in the future, so that the true role of the Protected Environment in the overall aggressive approach to cancer by chemotherapy can be clarified.

RADIATION THERAPY PROGRAM

The radiation therapy program consists of both research and training and is directed toward further development of radiation research. The major research activities are carried on by the cooperative investigative programs and in the specialized radiation therapy centers. Further activity pertinent to the development of these is the provision for exploratory studies in order to best assure quality in these activities. The present balance of these components of the radiation therapy program is not entirely appropriate, since, while research projects need to be supported at a fairly even level, the trend in support for training is sharply downward and the trend in center support and planning is definitely upward. Efforts of the Program Director have been focused primarily on planning and exploratory projects and, secondarily, on the evaluation of center programs and projects already under way.

Radiation Therapy Clinical Research Centers

Therapeutic radiology is the keystone in any system of cancer management. It is by definition a fulltime effort and interrelates with all the other medical and surgical specialties concerned with this disease. For these reasons it has been considered important to develop radiation therapy centers in the expectation that these might provide focal points around which comprehensive cancer management and investigation centers could be built. The growth of this program has been slow but relatively steady and there has been a recent increase in developmental and planning efforts. Although 11 radiation therapy centers are now in operation the growth of these centers should be accelerated. Since most research in the conquest of cancer takes place in such centers, to increase the quantity and quality of research, a considerably larger number of centers with more appropriate geographical distribution will be necessary. Many examples could be given; an outstanding one is the lack of radiation centers in either southern California or in the entire southern United States (with the exception of Houston). However, development of centers in some areas has been extremely rapid as in Boston where one large center is already funded and two more excellent ones are in the developmental stage and likely to be funded shortly.

Training in Radiation Therapy, Radiation Physics and Radiation Biology

Eleven years ago the shortage of trained radiation therapists was so acute the specialty could scarcely claim existence. At this time, the National Cancer Institute adopted a policy of special emphasis upon increased training in this field. From 10 such specialists in training in 1960, the number has grown to a level of almost 200. Approximately half of these are in training programs supported by the National Cancer Institute. The Committee for Radiation Therapy Studies, having surveyed the field in this specialty, expressed the opinion that there continues to be a critical shortage of specialists in light of the national needs. The support of the National Cancer Institute in this training endeavor has been vital for initiating and sustaining growth in the specialty, and at present the programs have not generally become self sustaining. Unfortunately, directors of training programs have not sought support for these activities from sources other than the National Cancer Institute. While some of these activities could and should be incorporated

into the functions of the research centers, this is complicated by the fact that centers now exist in only one third of the institutions with training programs.

Cooperative Clinical Studies

The growth of cooperative clinical studies has been gratifying, particularly in such diseases as Wilms' tumor in which no single center could ever hope to see sufficient numbers of patients to reach definitive conclusions about how the disease should be managed. Participation by radiotherapists in these programs has been steadily increasing and usually entry into such programs has been through the mechanism of single protocol projects or by cooperation with already existing and funded cooperative groups of chemotherapists or surgeons. In the past year a basic radiotherapeutic cooperative investigative group has been formed and the beginning of their activities funded. This group, the Radiation Therapy Oncology Group, is developing several protocols and has begun preliminary patient entry on two. The activities of this group will not supplant the previous concurrent cooperative efforts with long-standing cooperative groups in surgery and chemotherapy, as there are many problems needing investigation which are of concern solely to radiotherapists. It is expected that the Radiation Therapy Oncology Group will concern itself with such problems.

Research Grants

Radiation research in this category covers a wide spectrum of projects including effects of low levels of radiation, the basic mechanisms of damage and repair, and the problem of chemical modification of radiation response. Areas of major interest to be explored are (1) those pertaining to tumor recognition definition, and (2) the biological behavior of various forms of high LET radiation, some of which have been previously completely unexplored.

Exploratory Studies and Future Problems

The exploratory project mechanism has been extremely valuable in providing strength and support for the planning of major projects and centers. Considerable institutional commitment and support is often generated where it previously was lacking. This mechanism is also flexible, allowing for a considerable degree of channeling of efforts into areas of programmatic interest, such as the encouragement of planning for centers in areas where they are needed as well as the continuation of careful planning for large projects of major interest.

There are two primary areas of interest in the future development of the radiation program. One is the encouragement of support for radiation centers and, even more important, further efforts to encourage radiation centers to broaden their activities to include all cancer disciplines. The other major area of future interest developing during the current year has been that of particle irradiation. The use of charged particles for irradiation presented an intriguing challenge to the radiotherapeutic community. All radiobiological evidence available so far indicates that these types of beams obviate the problems of hypoxia which are believed to be at the root of radiation resistance in many tumors. Although this interest has been present for many years, such beams of radiation have not been available for cancer research because of the

cost of the machines which produced them, because the machines were utilized entirely for research in physics, and because they were located in relatively inconvenient places. Recently, there has been a drastic reduction in support for such activities from the Atomic Energy Commission and the National Aeronautics and Space Administration. The effect of this cessation of support has been to provide opportunities for exploring the biomedical and clinical effects of fast neutrons of various energies, proton beams, negative pi mesons, accelerated heavy ions, and alpha particles. A major task is the proper evaluation of the possibilities inherent in this situation and the careful exploration of therapeutic possibilities according to a rational system of priorities, not only among the various particles available, but with regard to the other activities of the radiation therapy program. Because some of these projects have been and are still being partially supported by the Atomic Energy Commission, the National Science Foundation, and the National Aeronautics and Space Administration, a liaison group of representatives from these agencies and several other Government agencies, including the National Cancer Institute, has been set up to meet monthly for review of these problems. Reasonable programmatic guidelines are expected by the end of the fiscal year.

IMMUNOLOGY PROGRAM

Because of the rapid advances in immunology -- especially in those areas applicable to the solution of problems in the diagnosis, treatment or prevention of cancer in man -- an Immunology Program was established in December of 1969. In addition to research grants bearing almost entirely on tumor immunology, several complex program project grants contain areas of high interest to the immunology program. Findings reported by investigators in these areas are continually integrated into the overall immunology program plan. The Program Director is especially concerned with the tumor immunology training grants which are considered to be a dynamic and integral part of the program. The fiscal year 1971 program is comprised of 63 research grants totalling $3,092,318, three program project grants totalling $1,279,474, and 14 training grants totalling $915,778. The total, $5,287,570, represents only a negligible increase over the fiscal year 1970 total of $5,285,600.

Within a realistically brief time, tumor immunology holds great promise for providing answers to some of the most compelling problems of oncology. Solid evidence now supports the fact that, associated with the cells of both virus-induced and carcinogen-induced tumors in experimental animals, there are antigenic determinants against which the animal mounts an immune response. Other evidence is now accumulating at a rapid rate that humans respond similarly to antigens of their own tumor cells. Such an immune response may or may not be manifested by tumor regression. It almost certainly is manifested by factors in the serum or cells, detection of which would be specifically diagnostic for malignancy. Elucidation of the myriad factors operating in this host-tumor relationship can lead to an understanding of how these immunological phenomena might be manipulated for the prevention, therapy or diagnosis of cancer in man.

One current finding which may lead to an easily mass-performed diagnostic test for certain cancers is that embryonic antigens are apparently present in the circulation of patients with incipient or frank malignancy. Several grants in the immunology program support much of this work and we eagerly await the research reports that will either sustain or refute the hope that this may indeed be a specific, reliable test. Because of the explosive interest in this area, the Program Director is currently planning a workshop to be held under the joint sponsorship of the Colorectal Cancer and the Immunology Programs.

The Program Director has spent considerable time visiting the sites of research, program project, and training grants concerned with immunology. After integration of the work supported under these grants with related work supported by other sources and available to the scientific community, it is clear that to accomplish program goals in minimal time, a concerted, organized effort will be required on the part of all concerned scientists. With this in mind, and under the aegis of the Immunology Program, a broad plan is being developed in which many able and interested investigators will be invited to participate. Because of the urgent need for knowledge in certain areas, attempts are now being made to encourage and expand

interest in them. For example, in order to plan human immunotherapeutic trials on a firm base, definitive studies in experimental model systems are mandatory -- very few of these have yet been done. Further, before any real advance can be made in understanding immunity to cancer in man, a reliable *in vitro* test correlating with the clinical status of the individual must be available. While potentially excellent tests are currently under scrutiny in several laboratories, there is as yet little agreement on their reliability and relationship to the clinical status of disease.

The predictable future contributions of immunology to the solution of problems of oncology seem immense and we look forward to the successful attainment of program goals.

SPECIAL PROGRAMS BRANCH
Cancer Research Centers

The Cancer Research Centers program was activated approximately a decade ago. As a result there is increasing cancer research, training and education being carried on in the medical schools, rather than being confined largely to specialized cancer hospitals and research laboratories. The complexities of the various neoplastic diseases and the need for an increased understanding of the fundamental processes involved require a coordinated effort on the part of scientists ranging from those undertaking basic research in cancer biology to research clinicians, and finally to clinicians responsible for the care and treatment of cancer patients. In order to achieve this, the medical schools, specialized cancer facilities, practicing physicians, and community medical services must participate in an integrated program. This will enhance the extension of the most advanced diagnostic and therapeutic techniques to all cancer sufferers.

The need to create new centers and strengthen or expand others continues. In this regard, geographic distribution should be an important consideration. As mentioned in last year's report, representation in the mountain and plains States still presents a problem. The National Cancer Institute currently supports 32 Specialized Research Centers, 12 of which are Radiation Centers. An additional institution was approved and will be funded as soon as budget negotiations are completed. Two applicants for center grants were disapproved during fiscal year 1971. The recommended support (direct costs) for the 33 approved centers for this fiscal year was $15.2 million.

The Cancer Research Centers are quite variable in their makeup. For this reason, it is not easy to make a comparison among them. Also, since they are for the most part functional rather than physical or organizational units, it is not always clear as to what degree certain activities are an integral part of the Center as opposed to other activities within the institution. A solution to the above might be to have a more centrally directed centers program. However, this could result in the loss of flexibility which allows for changes in centers programs and activities as new developments occur in cancer research and therapy.

The Research Centers provide a unique training ground for physicians, paramedical personnel, and students. Many of them are involved in the cooperative clinical trials of the NCI Clinical Investigations Branch. Others utilize their own therapy protocols. Since last year there have been no major changes in the direction of the centers' activities. As was indicated then, immunology is receiving greater attention. This should continue as more trained and experienced scientists become available in this field.

Twelve exploratory grant applications were approved this year. Although some have not yet been funded, it is anticipated that they all will be awarded. Two applications were disapproved. The objectives of this group of grants vary, but they primarily relate to planning for Research Cancer Centers, including Radiation. The recommended level of support for all types of exploratory grants for the fiscal year was approximately $1.5 million.

CLINICAL CANCER TRAINING PROGRAM

Clinical Cancer Training grants are awarded to medical schools, dental schools, schools of osteopathy, specialized cancer institutions, principal affiliated hospitals of medical schools, and schools of public health. The purpose of these awards is to encourage institutions: (1) to increase the quality of cancer instruction offered to undergraduate medical and dental students and to interns, residents and practitioners; (2) to broaden the scope of current cancer teaching; and (3) to seek new and better ways of providing clinical cancer instruction at one or more professional levels and of maintaining and evaluating the competence of those who provide clinical cancer management.

In 1970, there were 113 active Clinical Cancer Training grants distributed among 72 medical schools, 32 dental schools, 3 hospitals, and 6 specialized cancer institutes at a cost of $5,400,000. The distribution of grantee institutions by state is as follows:

Alabama	2	New York	13
California	9	North Carolina	4
Colorado	2	Ohio	4
Connecticut	1	Oklahoma	1
District of Columbia	5	Oregon	1
Florida	1	Pennsylvania	6
Georgia	3	South Carolina	1
Hawaii	1	South Dakota	1
Illinois	5	Tennessee	5
Indiana	1	Texas	4
Iowa	2	Utah	1
Kentucky	4	Vermont	1
Louisiana	3	Virginia	3
Maryland	3	West Virginia	1
Massachusetts	5	Wisconsin	1
Michigan	3	Puerto Rico	2
Minnesota	2		113
Mississippi	1		
Missouri	6		
Nebraska	2		
New Hampshire	1		
New Jersey	2		

While the Clinical Cancer Training grants provide opportunities for enriching the cancer teaching and training experiences of all undergraduate and graduate students in a grantee institution, stipends are also made available to selected trainees at various levels of their training. More than 350 trainees have been reported to date for FY 70. However, a total number of 600 is expected. The number of trainees reported since the inception of the program is 2,117, which is represented by the following:

Year	Medical Trainees	Dental Trainees
1966	181	2
1967	410	9
1968	560	18
1969	570	17
1970	332	18
	2,053	64

ADMINISTRATIVE:

Two training committees, one to consider only proposals from dental schools, were established in 1965 to provide technical assistance and advice on all applications, to make recommendations to the National Advisory Cancer Council and to the Director of the National Cancer Institute, and to maintain a continuing awareness of the needs for personnel and facilities in the field of cancer teaching.

The training committees have devoted considerable time to the development of criteria for the evaluation of proposals for training and for the assessment of ongoing Clinical Cancer Training Programs. The Program is continually being evaluated through project site visits and review and analysis of progress reports from the training institutions.

Funds appropriated for FY 1970 not only did not permit expansion of existing programs nor allow support of all new programs which were recommended for approval but required reductions in ongoing training. The plan to extend eligibility to additional teaching hospitals and encourage applications from new medical and dental schools has once again been deferred.

During FY 1970, new Guidelines were formulated by each of the Clinical Cancer Training Committees in order to define more clearly the purpose, goals, organization, administrative structure and characteristics of Clinical Cancer Training programs.

The Guidelines were approved by the National Advisory Cancer Council and will replace the "Information Statement" which inaugurated the Program in 1965.

Liaison has been established and maintained with other Federal programs such as the Regional Medical Program, and within the National Cancer Institute, close liaison is maintained with all related programs.

GRADUATE RESEARCH TRAINING PROGRAM

Awards in support of graduate research training are made to assist institutions in developing as well as in expanding training programs that are designed to encourage and aid medical and basic scientists to prepare for productive careers in academic medicine. These programs provide a source of competent research and teaching manpower that is directed toward alleviating shortages and maintaining a supply of skilled personnel in those areas of importance to the cancer problem.

Training grant support, paralleling research grant support, is distributed among the areas of cancer biology, carcinogenesis, cancer epidemiology, cancer immunology, cancer pharmacology, and radiation (comprising radiation therapy, radiobiology, medical physics, and nuclear medicine). Although the Institute continues to be assigned applications which have not been programmed, most of the proposals are generated as a result of staff contact with departments and institutions that have demonstrated an ability to develop and carry on vigorous and effective cancer training in those areas where there is a continuing need for trained professional personnel for teaching and research. Both Extramural Staff and Advisory Personnel contribute to the identification of specific areas for which the need for increased training opportunities is manifest as well as to the assignment of priorities among the areas that are in competition for funds budgeted for training.

In addition to providing the initial review of applications, the Cancer Research Training Committee maintains a surveillance of the accomplishments of ongoing programs in order to assess the progress being made and to determine whether the training on each program continues to be focused on the cancer problem. Additionally, the Committee has conducted studies relating to comparability of stipends and the supply of cancer personnel in selected areas. The Committee expressed concern that existing stipend ceilings are below the amounts paid by many universities, from their own funds, to predoctoral and teaching and research fellows, and by many hospitals to interns and first year residents. This becomes an important factor in recruiting since a candidate is often reluctant to accept an appointment as a trainee if the stipend he would receive would be considerably below that being paid to teaching fellows and residents with comparable amounts of training and experience. The Committee believes that ceilings should be revised to permit payment of stipend amounts that are comparable with stipends paid from sources other than training grants.

In providing an overview of manpower needs in specific areas, the Committee concerned itself with the personnel requirements that are consistent with good medical practice. The Committee believes that the need for medical oncologists is as great as that for radiation therapists. This is based on one medical oncologist consultant for every 100-200 new cancer patients. On the basis of from 500,000 to 600,000 new cases annually, the Committee estimates a need of from 4,000 to 5,000 medical oncologists. In order to encourage the training of more physicians in this area, consideration should be given not only to expansion of current training programs but also to establishment of new ones as well as to set more realistic stipend ceilings. Physicians using radiotherapeutic procedures rely heavily on radiation therapy physicists. Many

hospital radiation therapy departments have been unable to recruit an adequate number of well trained radiation therapy physicists. There should be 1 or 2 physicists for every 500 cancer patients treated annually. The supply is below the 360 professional physicists needed to work with 500 therapists and well below the future requirements of 900 when the number of therapists will have increased to 1,500.

The attention given to the various aspects of the cancer problem touches on the several areas of training. The importance of viral induction of tumors will be greatly magnified by the increased effort that is to be devoted to cancer virology. In order to mount a greatly expanded research program in cancer virology, there will be an enhanced demand for professional personnel who are well trained both in cancer biology and in virology. This will likely result in considerable pressure on existing training programs to expand in order to accommodate a greater number of trainees. The various components introduced into our environment that are suspected of having carcinogenic properties points out the need for more scientists trained in this area. It has been estimated that from 30 to 90% of cancer deaths can be avoided. Where these differences exist among migrants, by socio-economic status or by geography, the potential exists for finding the environmental elements that lead to these differences. Exploitation of this knowledge could lead to cancer prevention. It is important that the cancer epidemiologist, in cooperation with appropriate laboratory personnel, determine which of the environmental factors associated with carcinogenesis are controllable. This emphasizes the need for expanded cancer epidemiology training.

It is becoming increasingly apparent that most, if not all, tumors possess antigens which are foreign to the host in which the tumors are growing. Since immunological reactions against these antigens can affect the growth of the tumors as well as the morbidity or mortality of the hosts, the use of immune reactions against tumors pressages an important control mechanism. Additionally, these reactions will also become important in diagnosis. Although there are a number of experts in the individual fields of cancer and immunology, there is a marked shortage of trained people with a basic understanding of both cancer and immunology. The efficacy of chemotherapeutic agents in controlling specific types of neoplastic diseases is indicative that new compounds being synthesized or isolated will, as a result of appropriate testing, be shown to be effective against other tumors. In order to accomplish this with a minimum of delay, it will be necessary to train additional personnel who can synthesize and/or isolate therapeutic compounds and pursue basic and clinical pharmacologic investigations. The manpower pool of radiation therapists has been significantly increased as a result of the stimulus and support provided by the National Cancer Institute; however, the needs for personnel trained in this area are not being adequately met.

A survey of 442 former trainees who were in training 9 or more months during the period from 1 July 1967 through 30 June 1970 reveals that 130 continued training under support from other sources; 82 accepted positions at medical schools; 77 became affiliated with universities; 41 went on to complete residency training; 40 accepted positions in research laboratories; 36 received hospital appointments; 27 entered the Armed Services, and 9 elected private practice.

Approximately $5,250,000 of FY-1970 funds were awarded for support of 87 training programs. These funds were distributed among 57 institutions in 27 states and Puerto Rico. Training was provided for 700 stipendiaries as well as for a number of individuals who, while deriving considerable training benefits, received stipend support from a source other than training grants. Two programs also conducted training utilizing only FY-1969 funds. After 30 June 1971 support for 10 of the programs will be discontinued.

The FY-1971 budget for research training will provide support in negotiated amounts, both for continuing and for renewal programs, but will not provide support for a number of new programs that have been recommended for approval by the National Advisory Cancer Council.

RESEARCH CAREER PROGRAM

Research Career Development Awards are made to nonfederal public or private institutions to enhance the career development of young scientists with outstanding research potential or demonstrated capability for careers of independent research in the sciences important to the cancer problem. In June 1964, the National Institutes of Health discontinued Research Career Awards which were made to enable institutions to finance positions favorable to the intellectual growth and research productivity of established investigators of high competence. Existing awards are being funded.

Effective May 1, 1969, it was permitted to supplement Research Career Development Awards by grantee institutions from nonfederal funds in an amount compatible with the institutional salary scale. In all cases the total salary to be provided an awardee must be justified according to the guidelines for salary support, as stated in the policy brochures and as hereby amended.

The maximum NIH contribution to the salary support will remain $25,000 per year. Fringe benefits and indirect costs, where permissible, may be requested only on the NIH contribution. All other terms and conditions of the awards remain in effect.

If a grantee institution desires to contribute to the salary support of any of its Career or Development awardees, the following information must be included:

1. The amount of the proposed supplementation.
2. A certification that the supplementation is from nonfederal funds.
3. A justification of the <u>total</u> salary, not just the NIH contribution.

There has been established a third deadline for the submission of new and renewal Research Career Development Award applications - December 1. Under this deadline, applications will normally receive notification of action by the following July.

The review schedule for RCDA applications will therefore be:

Deadline	Notification of Action By
December 1	July
May 1	December
September 1	April

A policy change dated February 5, 1971, stated:

1. A continuing review of the NIH Research Career Development Award Program indicates that a limitation of five years' duration on these awards will cover the critical period of the career development of each supported investigator.

2. Therefore, the present practice of renewing these awards will be discontinued. For those individuals now participating in the program, renewals will not be made if the expiration date occurs on or after June 30, 1972.

During FY '71, 11 RCAs were funded at a cost of $331,057; 70 RCDAs at a cost of $1,686,389. These figures represent type 5 continuation commitments and six competing renewals awarded for one year only. In order to meet all commitments, the awards were held to the FY '70 level of funding.

As in FY '70, there have been no new awards made even though the National Advisory Cancer Council, at its November 1970 and March 1971 meetings, recommended 15 applications, including two competing renewals, for approval. Four were transferred to other Institutes and one was withdrawn. $215,890 would be needed to fund the remaining awards. There are three applications pending for the June 1971 meeting of the NACC. If these were recommended for approval and funded, the amount needed would be $49,525, making a total potential need in FY '71 of $265,415 for new awards.

There have been 18 terminations of RCDAs during FY '71:

9 came to the end of the award.
2 were not recommended for approval of the 5-year renewal.
1 5-year renewal received a priority too low to pay.
2 changed institutions.
4 received other support.

The bulk of the candidates were, as formerly, nominated from medical schools.

During FY '71, the percentage of candidates not recommended for approval decreased from 20% - 25% to 6% with a higher percentage of Ph.D.'s than M.D.'s.

Statistical analysis of geographic distribution shows that 24 states and the District of Columbia have awardees with the largest number in Pennsylvania:

Pennsylvania	15	New Jersey	2
Massachusetts	8	Oregon	2
New York	6	Arizona	1
Texas	6	Florida	1
Washington	6	Maryland	1
Wisconsin	6	Nebraska	1
Connecticut	4	New Hampshire	1
Illinois	3	Ohio	1
North Carolina	4	Oklahoma	1
California	3	Tennessee	1
District of Columbia	2	Utah	1
Kansas	2	Vermont	1
	Virginia 1		

Awardees of the NCI are currently involved in 15 primary scientific disciplines. The largest numbers are conducting investigations in biochemistry and virology, with immunology, chemotherapy and morphology-morphogenesis following closely. Other disciplines in which the awardees are involved include enzymology, radiology, endocrinology, genetics, physiology, epidemiology, chemistry, cytogenetics, histology-cytology, and historical medicine (carcinogenesis).

The number of applications for development awards assigned to the NCI during the past year has remained about the same as the previous year. The calibre of the candidates has remained high as evidenced by the lower rate of recommendations for disapprovals.

The continued inability of the NCI to fund any of the new applications recommended for approval by the NACC is indeed deplorable. It is hoped that this situation will be remedied in the near future.

Follow-up of the awardees whose awards have terminated shows that the major portion are still involved in research in the field of cancer. All of these individuals have expressed the opinion that the award was very valuable during tenure.

Efforts will be made to encourage more qualified young investigators to move into the areas of radiation therapy and cancer epidemiology which are shortage areas in this program.

FELLOWSHIPS PROGRAM

The fellowships program provides support to qualified American citizens for research training, to be obtained in this country or abroad, in the basic and clinical sciences associated with the cancer problem for the purpose of raising the level of competence and increasing the number of trained investigators and/or academicians in those biomedical sciences essential to the advancement of cancer research.

Postdoctoral support is offered to applicants who have earned a Ph.D., M.D., D.D.S., D.V.M., O.D., or equivalent degree. Special fellowships are offered to applicants who (a) have three or more years of relevant postdoctoral research experience, including residency or medical specialty training; (b) do not hold a doctoral degree but have demonstrated to the National Cancer Institute sufficient competence in their field to pursue their proposed training; or (c) who require special training, not provided by other programs, to meet particular program needs of the National Cancer Institute.

In the fiscal year 1969, the National Cancer Institute awarded 234 fellowships for $2,142,558, leaving unfunded 20 applications recommended for approval totalling $199,135. During fiscal year 1970, the Institute awarded 180 fellowships for $1,690,868, leaving 179 applications recommended for approval, at $1,640,718, unfunded.

The following sub-paragraphs reflect the character of the 1970 program:

Academic Level

Degrees held by fellows

Ph.D. - 138 D.V.M. - 3 (2 of these seek Ph.D.'s)
M.D. - 32 (7 of these seek Ph.D.'s) D.O. - 1
Six fellows hold two doctoral degrees

Fellows according to number of years past receipt of doctoral at time of application

No. of	1st yr.	2nd yr.	3rd yr.	4th yr.	5th yr.	6th yr.	7th or more yr.		Total Fellows
Fellows	33	76	12	5	10	7	37	=	180

(The large number 7 or more years past receipt of the doctoral, includes the M.D.'s who have completed residency training, and the senior investigators who are seeking special training during sabbatical leaves.)

Geographic Distribution

Training in the United States - 160 fellows, training in 71 different institutions, in 29 states as follows:

45%

17% in California for about 15% of the total funds
10% in New York for about 8% of the total funds
9% in Massachusetts for about 10% of the total funds
9% in Maryland for about 9% of the total funds

Training in Foreign Countries - 20 fellows, training in 15 different institutions, in 8 foreign countries as follows:

7%

6% in England for about 6% of the total funds
1% in Sweden for about 1% of the total funds

(The remaining 48% of the fellows trained were too widely distributed geographically to obtain significant percentages.)

Distribution By Discipline

The 180 awards represent training in 19 different disciplines, which fall into the broad program areas shown below:

Cell Biology - 80	Cancer Carcinogenesis - 52
Immunology - 13	Pharmacology - 22
Radiology - 8	Chemotherapy - 5

The Institute recognizes that experience gained through training in a foreign laboratory with an eminent scientist is extremely valuable. For this reason, fellowship requests for such training have had priority in the assignment of the limited funds allocated to the Institute under the current foreign ceiling. Most of the new awards for foreign training during the past year were in the field of radiology and radiation therapy. It is considered fortunate that the program has one fellow in England being trained in fast neutron radiotherapy, an area with not only a shortage of trained personnel but also with few institutions which can provide this training.

The Institute has continued to encourage radiology and clinical radiation therapy in domestic institutions. Despite the fact that the number of interested individuals who can qualify for fellowship support is limited, a program of training in radiation therapy is being maintained.

During the fiscal year 1969, about 60% of the total fellowship program represented training in some area of chemistry, i.e., biochemistry, pharmaceutical, medicinal, organic, or biophysical chemistry. Although the number of fellows currently being trained in this extensive field, which is so significant to cancer research and to cancer chemotherapy, has doubled since 1967, the current program is at about 35%.

The level of the 1970 program is currently about 45% of normal. It is expected that even this level cannot be maintained beyond fiscal year 1971. The rate of increase in the number of applications received is expected to accelerate in direct proportion to the diminution of research funds available to universities and research institutions.

However, the need for trained investigators does not remain static and should the opportunity arise, additional emphasis would be placed on expanding fellowship training in areas such as chemical and viral carcinogenesis, epidemiology and immunology.

CANCER SPECIAL PROGRAM
Single-Support Grant No. CA08748-05
(Sloan-Kettering Institute)

The Cancer Special Program Advisory Committee (CSPAC) has maintained a continuous review and evaluation of the single-support grant to the Sloan-Kettering Institute (SKI) during the past year. The review procedures followed those used in previous years so that information was further developed on (1) the quality and quantity of the research and training programs, (2) the morale and intellectual environment existing at SKI, (3) the scientific and fiscal administrative policies and practices at SKI, and (4) the capabilities of the SKI administration to develop and maintain a relevant and productive cancer research program. The progress of this continuous review and other matters was reported to the Director, NCI and the National Advisory Cancer Council in Progress Reports Nos. 10, 11, 12, 13.

In addition, the Committee developed a comprehensive 5th YEAR REPORT on the operation of this type of grant at the Sloan-Kettering Institute. This report is concerned with the considerations and decisions of the Committee on the functioning and value of the single-support granting mechanism at the SKI, and on the value of such a research-supporting device in advancing the mission of the National Cancer Institute.

The Committee concluded that this type of grant is particularly applicable to an institution whose activities are entirely devoted to one categorical disease such as cancer, and that this type of research support is of considerable importance in furthering the mission of the National Cancer Institute. The Committee, therefore, recommends that it should be recognized as an appropriate and workable mechanism to provide financial support for a total research program oriented to a categorical disease or a common research problem.

The Committee further recommends that (1) the grant be renewed at the SKI, if that institution wishes to continue this type of support, (2) a procedure be included in the terms of the new grant so that desirable fiscal adjustments can be made to each year's award (thus preventing the detrimental restrictions evident in the terms of the current grant), (3) before the renewal award is made the NCI Director be satisfied that the corrections to the deficiencies in the SKI internal review and evaluation capabilities, as recommended by CSPAC, have been corrected, and that (4) the NCI Director and Council explore the administrative structure and capabilities, and research capacities of other cancer research institutes with the intent of implementing the single support grant elsewhere.

The Committee intends to continue its comprehensive monitoring of the current grant during FY 1972, and to be available to the Director and Council for any review and advisory activities deemed necessary.

APPENDIX I

Published Results of Scientific Work

Supported by the Extramural Programs

of the

National Cancer Institute

July 1, 1970 through June 30, 1971

PUBLISHED RESULTS OF SCIENTIFIC WORK SUPPORTED BY THE EXTRAMURAL PROGRAMS OF THE NATIONAL CANCER INSTITUTE

Medical knowledge at times advances with giant steps that are quickly recognized and recorded in the pages of history. Each of these major "breakthroughs" is built upon many smaller steps which individually may be overlooked. One of the purposes of this report is to relate the small steps to each other so that cumulatively one may see, if not a giant step, at least a forward step. The history of cancer research indicates few if any spectacular advances. Most of the progress has been difficult and gradual.

This report, prepared by the Program Analysis and Reporting Section, OADEA, presents a selected portion of that hard gained knowledge which has been published by NCI-supported investigators, during the last twelve to fifteen months. The source of individual NCI extramural support for each paper cited appears as a notation with each reference in the form of the award number or numbers; also symbols of the extramural program areas are included.

CHEMICAL CARCINOGENESIS

An important aspect of chemical carcinogenesis is that most compounds must be metabolized by the host to a reactive form before their carcinogenic activity can be exerted. This fact accounts for much of the variation among species with regard to susceptibility to different chemical carcinogens. Correlation of species susceptibility with certain metabolic transformations of administered carcinogens has led to identification of the reactive metabolites of some compounds and has suggested possible mechanisms by which they might act. Evidence has been accumulated over the past several years which indicates that, despite their chemical dissimilarities, many carcinogens are metabolized in vivo to potent electrophilic reactants.[1] In no case, however, has it yet been possible to ascertain precisely how these reactive forms cause a cell to become malignant.

The most detailed knowledge concerning metabolic activation of chemical carcinogens which is currently available is that obtained from studies with aromatic amines. The carcinogenicity of certain aromatic amines and amides is known to require their N-hydroxylation. As a result of recent studies,[2] other structural requirements are beginning to emerge as determinants of the oncogenicity of N-acylarylhydroxylamines: the size of the aryl moiety (which must exceed a limiting size); and the position of the nitrogen (and therefore of the acyl group) relative to the aromatic system.

A third requirement which may affect the activities of these compounds is the cleavage of the acyl moiety from the

molecule in vivo. Thus, N-acylarylhydroxylamines in which the acyl moiety consisted of the acetyl, benzoyl, or benzenesulfonyl group, i.e., of hydrolyzable groups, were shown to be strongly oncogenic in rats. The low oncogenic activity of N-acetoxy-2-fluorenylbenzamide in this study appeared to contradict the hypothesis that esterification of a hydroxamic acid and subsequent interaction of the ester with tissue nucleophiles are required steps in the activation of arylamides. It is possible, however, that the extreme toxicity of the compound vitiated assessment of its carcinogenic potential by the oral route.

There is no evidence yet that N-hydroxy-2-acetylaminofluorene (N-hydroxy-AAF) can be enzymically acetylated to N-acetoxy-AAF. This reaction apparently can occur non-enzymically in the presence of acetyl-CoA.[3] In a study[4] designed to clarify the mechanism of the release of the N-acetoxy groups in the reactions of the N-arylacetamides with nucleophiles, ^{14}C-labeled acetic acid esters of four acethydroxamic acids were investigated as models of the reactive esters presumed to be formed in vivo, and the acetoxy group was shown to undergo nucleophilic displacement in aqueous media. Under the conditions of the experiment, however, the rate of loss of the N-acetoxy group did not always correspond to the extent of substitution by methionine or guanine. Recent studies[5,6] with N-hydroxy-AAF suggest that, at least in the liver, the sulfuric acid ester is the principal reactive and carcinogenic form. Evidence has been obtained that the highly reactive ester 2-AAF-N-sulfate is formed in rat liver in vivo. Injection of sulfate ion into rats given N-hydroxy-AAF increased (1) the formation of 1- and 3-(methion-S-yl) 2-AAF bound to protein in the liver, (2) the formation of fluorenyl derivatives bound to total protein, rRNA, and DNA in the liver, and (3) the toxicity of the carcinogen. The close correlation observed between N-hydroxy-AAF sulfotransferase activity and the amount of reactive esters formed in vivo also strongly implicates AAF-N-sulfate as an important reactive form in vivo. Furthermore, the correlation of these two parameters with the various factors known to affect the carcinogenicity of N-hydroxy-AAF in rat liver suggests at least that AAF-N sulfate is one of the major ultimate carcinogenic metabolites of N-hydroxy-AAF in this tissue.

It appears unlikely that 2-AAF-N-sulfate is involved to a significant extent in the binding of N-hydroxy-AAF to rat liver nuclear DNA in vivo; the glucuronide appears to account for most of the DNA binding observed.[7] The sulfate ester, or related reactive esters, might, however, be involved in the binding of N-hydroxy-AAF to cytoplasmic nucleic acids or proteins.

Like the acetate and sulfate esters, AAF-N-phosphate can react in vitro with methionine and guanosine.[8] The ring-phosphate is unreactive under similar conditions. AAF-N-phosphate might be formed in vivo by the interaction of N-acetoxy-AAF and P_i or by enzymic conversion of N-hydroxy-AAF in the presence of

ATP and Mg^{2+} ions. The behavior of 3-hydroxyxanthine in vivo and the reactions of 3-acetoxyxanthine in vitro resemble the metabolic reactions of carcinogenic arylhydroxylamines, the reactivity in vitro of their o-acetyl derivatives, and the possible formation in vivo of sulfate or phosphate esters. Thus, the excretion of 8-substituted chloro- and methylmercapto- derivatives by rats treated[9] with 3-hydroxyxanthine could be explained by conversion in vivo of 3-hydroxyxanthine to an ester with reactivities similar to those of 3-acetoxyxanthine.

Another potential metabolite of N-acetyl-N-2-fluorenylhydroxylamine has been synthesized and characterized.[10] This type of glucuronide, sodium (N-2-fluorenylhydroxylamine β-D-glucosid) uronate (N-GIO-AF), has not previously been reported in the literature. It is quite unstable in aqueous systems and binds covalently to guanine residues of RNA and DNA in vitro at a rate much faster than that previously reported for sodium (N-acetyl-N-2-fluorenylhydroxylamine β-D-glucoside)uronate. N-GIO-AF may be formed as an intermediate metabolite of N-acetyl-N-2-fluorenylhydroxylamine, thus accounting, at least in part, for the observed binding of the carcinogen to rat liver DNA in vivo.

Studies have been undertaken[11] to determine the mechanism by which attachment of AAF to guanosine residues in nucleic acids distorts their structure and function. Oligonucleotides were modified with N-acetoxy-AAF, repurified, and their base compositions analyzed. AAF residues bound to guanosines in GpUpU, ApApG, or poly (U,G) appear to inactivate their function in codon recognition as assayed by the ribosome binding technique. Circular dichroism spectra suggest that this is caused by gross conformational changes in these compounds involving both a rotation about the glycosidic bond of guanosine residues bearing AAF, as well as stacking interactions between the drug and bases adjacent to the substituted guanosine. The relationship of these findings to the carcinogenic activity of AAF is not known. If attack on the DNA, with subsequent somatic mutation, is the critical event, then a conformational change would be more likely to produce small deletions rather than single base changes during DNA replication. A conformational change in specific regions of the DNA might also impair the transcription of certain genetic loci. An AAF-induced conformational change in tRNA would explain the previously observed[12] inhibitions of tRNA aminoacylation and codon recognition.

Because of the extremely low mitotic index in normal rat liver and the low level of binding of carcinogens, it has not been possible to compare the amounts of chemical carcinogens bound to liver DNA of resting and replicating cells in vivo. However, the question of whether N-hydroxy-AAF was bound to rat liver DNA only or primarily during replication has been studied[13] in vivo by administering bromodeoxyuridine (BUdR) to label newly synthesized DNA and then determining whether the carcinogen was bound to un-

replicated or BUdR-containing DNA after isolation from the liver after sacrifice. The results clearly showed that N-hydroxy-AAF does not bind to DNA exclusively or predominantly during replication.

Spectrophotometric and sedimentation analysis and electron microscopic examination have revealed[14] the presence of several altered properties in DNA from premalignant hyperplastic nodules in the livers of rats fed AAF, but not in DNA from normal liver or from the surrounding non-hyperplastic liver tissue. The altered DNA in the non-hyperplastic cells, containing scattered sites of arylation with no disorganization of the secondary structure of the double-stranded DNA, is presumably enzymatically repaired in such a manner as to yield essentially normal liver nuclear DNA. In contrast, in the proliferating cells, it is possible that the DNA has replicated before sufficient time or other conditions have allowed full excision and repair of the damaged DNA. The DNA altered by the carcinogen had (1) an altered absorption spectrum in the range 350-310 mμ, (2) a small population with an increased buoyant density as observed in equilibrium centrifugation in CsCl, and (3) denatured and distorted fibers as observed with electron microscopy. These findings suggest that an observable alteration in DNA may play a role in carcinogenesis and suggest a possible mechanism for the progressive nature of the cellular changes during cancer induction. The presence of the aryl group at certain sites could conceivably induce DNA polymerase to replicate distorted or branched molecules rather than the normal long duplex strand of DNA. Once initiated, the branching and distortion of the DNA could be expected to become increasingly greater and could account for the stepwise appearance of the properties characteristic of advanced malignancy.

Further support for the concept that carcinogenesis may depend on replication of damaged DNA comes from experiments involving croton oil promotion of skin carcinogenesis in mice. At a level which is effective as a skin tumor promoter, croton oil increases the rate of incorporation of tritiated precursors in skin DNA, RNA, and protein.[15] The promoting action of croton oil can be suppressed[16] by hydroxyurea, which is lethal to cells undergoing S phase DNA synthesis but which does not inhibit DNA repair synthesis. The hydroxyurea may thus afford the cells an opportunity to repair the DNA damage caused by the initiating carcinogen before the promoter stimulates replication. Preliminary findings[17] suggest that the reactive metabolite of hydroxyurea is N-carbamoyloxyurea.

A number of polycyclic aromatic hydrocarbons bind to the double-stranded, acid form of polyadenylic acid. Model building shows[18] that these hydrocarbons may intercalate in the helix, and be well protected from contact with the aqueous medium. Hydrocarbons that are too large to be so protected are found not to bind. A

size criterion for the binding of hydrocarbons to poly A therefore exists and differs from the one previously found[19] for DNA. In the latter study, the binding of 14 polycyclic aromatic hydrocarbons to DNA was tested; predictions based on the size criterion were found to be valid in all cases. The size criteria for DNA and poly A, together, serve as strong evidence for the intercalation model for hydrocarbon complexes. There can be little remaining doubt that intercalation is the mode of binding. Hydrophobic interactions, at least to first approximation, appear to dominate the binding.

Both natural and synthetic DNA's bind to microsomal membrane and the resulting complex can be detected by CsCl density gradient centrifugation. The density of the complex and, in some cases, the amount of nucleic acid associated with rat liver cell membranes has been shown[20] to be changed in the presence of various chemical carcinogens. The more reactive metabolites of the carcinogens had a distinct tendency to increase the amount of nucleic acid bound to the membrane. Alterations of the plasma membrane during malignant transformation make plausible the suggestion that the observed reactions are involved in the carcinogenic process. Moreover membrane changes may directly affect DNA synthesis since studies[21] of DNA synthesis in regenerating rat liver indicate that DNA replication is initiated at the inner layer of the nuclear envelope.

Because the physicochemical and biological effects of actinomycins have been studied extensively and since actinomycin D acts by a specific molecular mechanism, this drug may be useful in examining the role of DNA binding in the initiation of neoplasia. Multiple intraperitoneal injections of actinomycin D have been shown[22] to induce invasive, transplantable tumors (possibly mesotheliomas) in rats. Actinocylgramicidin S, which does not bind DNA, but which possesses the universal actinomycin chromophore, was not carcinogenic in these experiments. The results of another study,[23] in which rats were given intravenous or intraperitoneal injections of actinomycin D, indicate that there exists a hierarchy of binding sites for the drug, dictated in part by the functional state of the chromatin.

DNA is by no means the only cellular macromolecule of importance in the binding of chemical carcinogens. Hydrocarbons are bound covalently to a specific soluble protein in a direct relationship to their carcinogenic activities;[24] their covalent binding to DNA is less well correlated with carcinogenicity.[24] Extensive, but not complete, purification of the mouse skin protein to which the hydrocarbons bind has been accomplished.[25] The protein has been termed skin h protein because of its similarities to the liver h protein to which hepatocarcinogenic aromatic amines are bound. Although the principal soluble protein conjugates of three classes of chemical carcinogens (azo

dyes, aromatic amines, and polycyclic hydrocarbons) are of closely similar charge, it now appears[26] that they differ considerably in molecular weight. The liver protein which binds dimethylaminoazobenzene has recently been shown[27] to be identical with "cortisol metabolite binder I," one of 4 macromolecules in liver cytosol that bind anionic metabolites of cortisol. This demonstration provides, for the first time, evidence that carcinogens and corticosteroids have an identical site of action at the molecular level.

A number of arylcysteine derivatives, metabolites of polycyclic aromatic hydrocarbons, have been synthesized and tested for their ability to act as substrates for aminoacyl-tRNA synthetases in an in vitro system.[28] They were observed to compete with a variety of natural amino acids, and the structure of the hydrocarbon moiety conjugated with the sulfhydryl group of cysteine determined in particular the synthetases involved in amino acid activation. Moreover, none of the cysteine conjugates was activated by the cysteinyl-tRNA synthetase. Hydrocarbons are bound to protein by this pathway at sites different from those resulting from direct interaction. Incorporation into a protein by this pathway must be a rare event, however, because the level of activation and transfer of the conjugates was less than that for the natural amino acids. Also, the principal reactions of carcinogenic hydrocarbons in tissues are most likely the detoxication processes of mercapturic acid and phenol formation.

The somatic mutation theory of cancer has always occupied a central, although controversial, position in fundamental cancer research. Evaluation of the problem has been handicapped by lack of information regarding the chemical bases of both biological phenomena. Recent advances in molecular biology have made possible the interpretation of induced mutations in terms of chemical changes in DNA structure, either as a result of direct attack by mutagenic agents or through some indirect interference during its replication or repair. Furthermore, the finding that most organic chemical carcinogens are metabolized to ultimate carcinogenic forms has necessitated a reevaluation of many past investigations of the possible correspondence between mutagenic and carcinogenic activities of chemicals. It has been shown,[29] for example, that the sulfuric acid ester of N-hydroxy-4-acetylaminobiphenyl and the acetic acid esters of N-hydroxy-4-acetylaminobiphenyl, N-hydroxy-4-acetylaminostilbene, and N-hydroxy-2-acetyl-aminophenanthrene can cause mutation as well as inactivation of Bacillus subtilis transforming DNA in vitro. The parent amides, amines, N-hydroxy-amides, and N-hydroxy-amines, on the other hand, caused little or no inactivation or mutation under similar conditions.

N-methyl-N'-nitro-N-nitrosoguanidine (NG), a powerful mutagen in bacterial systems, produces carcinoma of the glandular stomach

and duodenum in rats.[30,31] Aflatoxin, which is one of the most potent rat liver carcinogens known, is also mutagenic in bacteria,[32] causing single base pair changes as well as what are apparently small deletions.

Evidence of deletions has also been observed in Drosophila after treatment with various compounds which are carcinogenic in mammals. The cytotoxic and mutagenic activities of urethane and two of its metabolites, N-hydroxyurethane (NHU) and N,O-diacetyl-N-hydroxyurethane (DAHU) were compared[33] after injection of the compounds into the hemocoel of adult male Drosophila. Decisive mutagenicity was indicated by small chromosome deletions manifested as minutes; with urethane and NHU, this result was restricted to metabolizing cells, but extended to mature sperm with DAHU. Chromosome breakage by urethane has been attributed[34] to in vivo conversion to NHU, which could then initiate main-chain scission in the DNA, through attack by OH or other oxidizing radicals. Clearly, this is not the mechanism involved in the cytotoxicity or mutagenicity of urethane in Drosophila where the carbamate and NHU were equally active.[33] A crucial step in the metabolic pathway of urethane activation during mutagenesis as well as carcinogenesis might involve the esterification of the hydroxamic acid into the o-ester: the oxyacetyl (as in DAHU), the glucuronide, the phosphate, or the sulfate.

Mutability tests in Drosophila were extended[35] to include various dimethylbenz[a]anthracenes. Particular derivatives were found to be active on specific gene loci involved in transcription of the various categories of RNA: on the nucleolar organizer (ribosomal RNA) segment, leading to bobbed (bb) mutations and on the presumed loci for transfer RNA, producing minutes. Experiments designed to test simultaneously specific mutability at the bb locus and some classical euchromatic loci demonstrated[36] that the tested polycyclic aromatic carcinogens, although inactive on the euchromatic loci, all induced deletions in the heterochromatic nucleolar organizer region, resulting in bb mutations. Significantly, the mutagenic activities of the tested compounds on the nucleolar organizer segment appeared to correlate satisfactorily with their carcinogenicity in rodents.[35] The type of deletions produced by these carcinogens would be expected to result in disturbances in the balance among the various cellular RNA components, leading in turn to defective protein synthesis or to the absence of specific proteins in accordance with the "protein deletion" interpretation of carcinogenesis.

Further testing of the mutagenicity of representatives of other classes of chemical carcinogens has now been carried out.[37] Mutations arising from direct intramolecular DNA damage, such as point mutations and chromosome breaks leading to viable rearrangements, occurred only with the alkylating agents and the nitroso compounds; urethan and its metabolites were also

weakly effective in inducing point mutations in mature sperm. All the tested compounds induced small chromosome deletions resulting in minutes, and a suggestive correlation between this function and carcinogenicity seemed to occur both between and within the various chemical series. The induction of these deletions with the hydrocarbons and aromatic amines and, in part at least, even with the alkylating carcinogens, appeared to be indirect; this was probably due to the *in situ* interference with the reproduction or repair of the DNA. In the light of this study, chemical carcinogens can be classified into two well-defined groups on the basis of their mutagenicity: (a) gene mutators and (b) gene inactivators. Group (a) includes alkylating agents, nitro compounds, and lactones; the latter two produce alkylating radicals in vivo. Group (b) includes hydrocarbons, aromatic amines and probably urethan and its derivatives.

In contrast to the rather specific mutagenic activity demonstrated in the work cited above, mutagenicity studies[38] of chemical carcinogens in bacteriophage T4 indicated that if a compound is carcinogenic because it can induce mutations, any type of gene-inactivating mutation is likely to suffice. Nine carcinogens tested were toxic to intracellular T4 but were not mutagens. Four of these were carcinogenic inorganic salts. Manganese, apparently not a carcinogen, is a potent mutagen in bacteria and phage. Results with the six chemically reactive carcinogens showed no correlation between the effectiveness of a compound as a mutagen in this system and its potency as a carcinogen, and no single characteristic type of mutation was induced by the six compounds. Unfortunately, even a completely consistent correlation, whether positive or negative, would not constitute a final proof of whether or not the mechanism of chemical carcinogenesis involves a somatic mutation. Experiments designed to test for carcinogenicity and mutagenicity in the same organism would, however, provide more insight into this problem.

VIRAL CARCINOGENESIS

Recognition of the importance of cell membrane alterations in malignant transformation has led several groups to investigate the effect of oncogenic viruses on membrane components and functions. The remarkably coincidental changes in cell morphology and in uptake of certain sugars observed[39] in chick embryo cells infected with Rous sarcoma virus suggest that both types of change may be related to a functional modification of the plasma membrane. Sugar transport efficiency could be changed drastically in response to a rearrangement or allosteric transition of membrane subunits; such changes in the cell membrane may well be correlated with the appearance of the transplantation neoantigen located on the surface of the transformed cells. Parenthetically it should be noted that the increase in sugar uptake

can be used as a more precise quantitative index than cell morphology to measure cell transformation; in this system, uptake of 2-deoxy-D-glucose gives the most reliable estimate.

Structural changes in the surface membrane, which can be detected by reaction with phytoagglutinins, are characteristic of cells transformed by, or productively infected with, wild type polyoma virus. These surface changes fail to occur in mouse cells infected by mutants of the virus which are unable to cause transformation.[40] Induction of agglutinability by the wild-type virus can be prevented by ultraviolet inactivation of its DNA, showing that gene function by the virus is required for this effect. There is also a requirement for DNA synthesis in order for the membrane change to occur in cells infected with the wild-type virus, but the requirement seems to be for cellular rather than viral DNA synthesis. Moreover, stimulation of cellular DNA synthesis is not in itself sufficient to cause the membrane change or to transform the cells since the nontransforming polyoma mutant NG-18 is capable of inducing cellular DNA synthesis in this system. The uncoupling of DNA induction from the surface alteration by this mutant suggests either that some aspect of DNA stimulation by the mutant is faulty or that two distinct viral functions are involved.

Other mutants of polyoma virus have been isolated in which transforming ability is temperature dependent. Incubation at 32° C of BALB/3T3 mouse cells infected with the mutant ts-3 causes appearance of phytoagglutinin receptors at the cell surface but no such change occurs following infection and incubation at 39° C.[41] In the same study, the ts-3 mutant was used to transform baby hamster kidney (BHK) cells. The ts-3-transformed BHK cells also showed enhanced agglutinability when grown at 32° C, but not when grown at 39° C. The surface properties of these cells at the higher temperature were similar to those of normal cells, in that enhanced agglutinability could be produced by mild protease treatment. Shifting the BHK cells from 32° to 39° C caused masking of the phytoagglutinin receptor sites beginning at 4 to 8 hours; masking of these sites was essentially complete by 24 hours after the shift. Exposure of the receptor sites after a shift from high to low temperature was not detectable by 12 hours after the shift but was substantial by 24 hours, and was essentially complete by 36 hours.

Several years ago a polyoma virus mutant was described which could transform BHK cells at 31° but not at 38.5° C.[42] Once transformed, however, the cells maintained their transformed phenotype and did not produce virus even when shifted to the higher temperature. The same mutant, designated Ts-a, has now been studied in 3T3 cells.[43] In these cells, maintenance of the transformed phenotype requires that the Ts-a function be inoperative; if the Ts-a function is expressed, as it is at 31° C, replication of the viral genome is induced. The characteristics

of these Ts-a-transformed 3T3 cells are of special interest because they are the first transformed lines in which virus multiplication can be activated in a large proportion of cells under controlled conditions and without cell fusion. These are also the only transformed lines in which a viral genetic function has been shown to have a role in the activation of viral multiplication. The physical state and replication of the Ts-a DNA following the shifts of transformed 3T3 cells (Ts-a-3T3) from 38.5 to 31 °C was investigated in detail.[44] It was shown that following activation at 31 °C, the cultures continued to synthesize viral DNA for at least 24 hours after they were shifted back to the higher temperature. The results of this study suggest that induction of viral multiplication in these cells involves the asynchronous occurrence of a unique event, after which DNA synthesis can continue even under non-permissive conditions. This investigation also disclosed that superhelical closed circular dimers and trimers as well as monomers of viral DNA were synthesized after the transformed cells were shifted to 31 °C. The apparent absence of appreciable viral DNA oligomers in acutely infected 3T3 cells suggests that neither replication of Ts-a DNA *per se*, nor recombinational processes in Ts-a3T3 cells, can account for the occurrence of circular oligomers after activation. The oligomers might arise from replication of multiple viral genomes integrated in tandem in the host cell chromosome. Alternatively, multiple copies of the viral DNA might exist in the cell as autonomous non-integrated structures, replication of which would be limited at nonpermissive temperatures to a rate just sufficient to maintain several copies per cell. Oligomers which might form infrequently at high temperatures, by recombination or other means, would then be produced in large numbers at permissive temperatures by normal replication of existing oligomers.

Analysis of DNA synthesis in cells infected with another of the papova viruses, SV40, has demonstrated the incorporation of cellular DNA into SV40 capsids.[45] These particles, known as pseudovirions, were detected only in primary African green monkey kidney (AGMK) cells but not in BSC-1 or CV-1 monkey kidney cell lines. In an effort to determine why SV40 pseudovirions were not produced in these permanent cells lines, the events occurring during lytic infection of AGMK, BSC-1 and CV-1 cells were compared. After infection of CV-1 and AGMK cells with SV40, the rate of cellular DNA synthesis was found to increase 8 to 10-fold.[46] No induction of cellular DNA synthesis was detected in BSC-1 cells. Between 13 and 23% of the AGMK cell DNA was found to be excised to small molecular weight fragments by 96 hours after infection. In contrast, excision or fragmentation of cellular DNA did not occur in BSC-1 cells and was barely detectable in CV-1 cells. These results indicate that AGMK and CV-1 cells contain a cellular component that is required for the virus-directed induction of cellular DNA. DNA preparations from

isolated nucleoli of CV-1 cells contain a dense satellite band not present in BSC-1 cells,[47] which may be relevant to the problem of induction of cellular DNA synthesis. Satellite DNA is the first cellular DNA to be synthesized in mouse kidney cell cultures after infection with polyoma virus.[48]

Induction of cellular DNA synthesis by oncogenic viruses facilitates the integration of viral genetic information into host cell DNA which leads to malignant transformation of the cell. If integration occurs in ova or sperm, the viral genome could be transmitted to offspring in a manner similar to that in which host genes are transmitted. Although mammalian ova have been infected with viruses, there has been no direct evidence of viral infection of mammalian spermatozoa. An attempt to infect rabbit spermatozoa with SV40 was unsuccessful.[49] When SV40 DNA was substituted for whole virus, however, successful infection was achieved.[49] Ova fertilized with the infected sperm cells were subsequently cocultivated with CV-1 cells, which are permissive for SV40 replication, and infectious SV40 was recovered. This provides the first direct evidence that a heterologous genome can be incorporated into a mammalian spermatozoan and subsequently transmitted to an ovum during fertilization.

A persistent problem with regard to the RNA tumor viruses has been the mechanism whereby they induce the heritable alterations in their host cells which are expressed as malignancy. A partial solution has finally been provided in simultaneous reports by two groups[50,51] working independently of each other. Evidence was presented for the existence of an enzyme, apparently associated with the virion, which synthesizes DNA on an RNA template. This "reverse transcriptase" was originally found in Rous sarcoma virus[50,51] and Rauscher murine leukemia virus[51] and has subsequently been found associated with all other oncogenic RNA viruses examined.[52]

The ability of an RNA virus to serve as a template for the formation of a DNA "provirus" would provide an obvious mechanism for integration of viral information into the DNA genome of the host cell. That provirus formation was necessary for cell transformation had been inferred from the fact that transformation by Rous sarcoma virus could be suppressed by selectively inactivating the DNA synthesized within 18 hours after infection.[53] This was achieved by treating the infected cells with bromodeoxyuridine during this period and later exposing them to visible light. Further support for the role of the DNA provirus in RNA viral carcinogenesis was provided by studies[54] which showed that a Rous sarcoma virus mutant which lacked DNA polymerase activity was noninfectious. Infectious pseudotypes which contain the mutant genome can be produced in the presence of a helper virus. Cells transformed by this pseudotype are indistinguishable from cells transformed by other Rous sarcoma virus strains in all biological characteristics except that they produce noninfectious

virus particles.[54] It appears, therefore that the DNA polymerase is not necessary for either the maintenance of transformation or virus replication. The polymerase provided by the helper virus is presumably necessary only for production of the mutant provirus which is inserted into the cellular genome.

The DNA polymerase(s) of Rous sarcoma synthesizes two principal products: single-stranded DNA in the form of a DNA:RNA hybrid and a double-stranded DNA. All of the single-stranded product[55] and 50% of the double-stranded product[55,56] can be hybridized to the viral RNA. The viral RNA apparently is utilized as a template by the RNA-dependent DNA polymerase, resulting in the formation of the DNA-RNA hybrid[57] which then serves as a template for the formation of double-stranded DNA.[58] The second reaction is DNA-dependent[58-60] and can use either double-stranded DNA or a DNA-RNA hybrid as template. It is the first DNA-dependent DNA polymerase found which prefers double-stranded rather than single-stranded template DNA.[58] An endonuclease detected[60] in Rous sarcoma virions may account for the fact that the double-stranded DNA molecules transcribed by the two polymerases are so much smaller than the viral template RNA. The endonuclease cleaves exogenous, as well as virus-specific, DNA and may therefore play a role in the integration of the provirus into the cell genome. In the presence of an ATP generating system endonuclease activity is reduced but no reduction in activity occurs in the presence of the dNTP alone, suggesting that in addition to the endonuclease there may also be a DNA ligase present in the virion.

The DNA-dependent DNA polymerase activity induced by another RNA-containing avian tumor virus, MC29, has been shown to be associated with the virus fraction from chick embryo cell cultures infected with MC29.[61] The difference in kinetics of DNA synthesis shown by the enzymes from infected and control cultures strengthened the association of the enzyme activity with virus infection. The virus-induced polymerase activity was detected within 48 hours after infection, the earliest time period yet investigated. Plasmas from two patients with chronic lymphocytic leukemia were examined[62] in an attempt to detect a similar polymerase activity in human leukemic cells. In both cases detectable enzyme activity was present. Enzyme activity in the plasma pellets was increased by detergent treatment, implying that the enzyme was bound in a particulate and not available to the substrates in the reaction mixture until released by the detergent treatment. Absence of particulate enzyme activity in plasma from healthy persons demonstrated that the activity in the leukemic plasmas was not a measure of low enzyme activity normally present in plasma.

In the few months since the discovery of new DNA polymerase activities in RNA tumor virus-infected cells, there have been several findings reported which necessitate reappraisal of the original interpretation of this discovery. The first is that Rous sarcoma

virus, previously believed to contain only RNA, contains a small amount of DNA as well.[57,63] The two components of the DNA obtained from the virion are reported[63] to sediment at approximately the same rate as the two DNA products synthesized in vitro by the RNA-dependent DNA polymerase. The viral DNA is believed to be located in the interior of the particle and is therefore probably not an artifact of the adsorption of cellular DNA to the virion. The virion DNA may be the result of abortive synthesis during maturation of the virus, but it could represent either the template for the DNA-dependent DNA polymerase or cellular DNA incorporated into the virion during maturation.

The hope that presence of the novel reverse transcriptase would prove pathognomonic of infection with an oncogenic virus was weakened by the finding of such an enzyme in virions of visna virus[64-66] and primate syncytium-forming (foamy) virus[67] neither of which is known to cause tumors. Nevertheless, failure to display oncogenic activity can hardly be accepted as decisive evidence of its absence, and visna virus has many other similarities to known RNA tumor viruses with respect to size, virion structure, site of maturation, sedimentation coefficient of its RNA, etc. Its reverse transcriptase could therefore still be considered indicative of its oncogenic potential were it not for the fact that the previously elusive enzyme is rapidly becoming almost ubiquitous.

In addition to being associated with several presumably noncarcinogenic viruses, RNA-dependent DNA polymerase has now been found in several types of apparently normal uninfected cells. The first non-malignant cells in which the enzyme activity was demonstrated[68] were cultured mouse 3T3 cells, human skin fibroblasts, and human lymphocytes. No activity could be detected in crude extracts from the uninfected cells when a double-stranded synthetic RNA template was used, but use of an RNA-DNA hybrid resulted in levels of DNA synthesis comparable to those in transformed cells under similar conditions. After partial purification, the enzyme from the normal cells was also able to transcribe the rA strand of an rA·rU template. Some DNA synthesis was obtained from a dAT template but rA·dT was still the most effective template even with the partially purified enzyme.

The finding[68] of an activity in extracts of normal mammalian cells which can transcribe the RNA strand of poly rA·dT and the rA strand of rA·rU is analogous to the observation that the otherwise DNA-dependent DNA polymerase from E. coli can, under certain conditions use either synthetic[69] or naturally occurring[70] RNA's as templates for DNA synthesis; the first product of such a reaction is an RNA-DNA hybrid, which is followed by the formation of double-stranded DNA.

In a more recent report[71] concerning the occurrence of reverse transcriptase in normal cells, complete separation of the DNA-

dependent and RNA-dependent activities was achieved. RNA-dependent DNA synthesis was observed in phytohemagglutinin-stimulated normal human lymphocytes from six healthy donors. After electrofocusing, the RNA-dependent activity in these cells was in some cases of the same order of magnitude as the DNA-dependent activity, perhaps indicating a major role for this enzyme in normal lymphocyte function. At the present time, however, the possible presence of a latent virus cannot be excluded.

A hypothesis which would account for the presence of reverse transcriptase in normal cells while still linking this enzyme to malignant transformation has been proposed.[72] According to this hypothesis, RNA tumor viruses arise from "protoviruses" which are postulated to be normal cell components which transmit information from DNA through RNA intermediates back to DNA, and which are capable of passing from cell to cell in an organism. Protoviruses would not have an envelope like RNA tumor viruses. In normal development, the DNA protovirus in one cell would act as a template for synthesis of an RNA copy, which would enter another cell and act as a template for synthesis of a DNA copy which would become incorporated into the genome of the second cell. These protoviruses would contain genes for their own replication and for control of cell multiplication. If the free DNA form of a protovirus happened to integrate into a region of the genome controlling membrane proteins, the genome of an RNA tumor virus could be formed. This hypothesis differs from the oncogene theory[73] in that the latter proposes that the information for complete tumor viruses must preexist in all cells, and that the appearance of these viruses results from a nongenetic change such as derepression.

The evidence that any type of human cancer is caused by a virus is so far only circumstantial. The most recent evidence derives from a case[74] in which a girl with acute lymphoblastic leukemia was given an infusion of marrow from an HL-A matched brother following whole-body irradiation. Recurrent leukemia was evident 62 days after the successful marrow graft. Cytogenetic studies showed that the leukemia recurred in XY, presumably hitherto normal, donor-type cells. Although the mechanisms underlying this development are not known, a likely possibility is activation of a leukemogenic agent, such as a virus, in susceptible cells, or transfer of such an agent from host to donor cells.

CANCER BIOLOGY AND BIOCHEMISTRY

Cell suspensions from carcinogen-induced hyperplastic nodules in rat liver grow well in vitro, whereas cells from adjacent liver or from livers of untreated rats do not grow under comparable conditions.[75] Hepatocarcinoma cells induced by the same carcinogens grow readily in vitro but behave differently from nodular cells. Cultures of "premalignant" cells capable of growth in vitro will be extremely

valuable in the study of liver carcinogenesis if it can be demonstrated that these cells are indeed hepatocytes; morphologic evidence indicates that they may derive from liver parenchymal or ductular cells.[75] Experiments to determine whether abnormalities of enzyme regulation are already present prior to recognizable malignant transformation revealed[76] that the corticosteroid-mediated enzyme regulatory mechanisms were intact in 2-acetylaminofluorene-induced hyperplastic nodules. Although the specific activities of the enzymes studied were lower in the nodules than in the controls, the percentage induction of enzyme activity was the same as in the controls. The most severe depression of enzymatic activity occurred before the appearance of large nodules in the males, and was equally severe in males and females, although the females are far less susceptible to the carcinogenic action of 2-acetylaminofluorene. Failure to induce enzymatic adaptive responses at this stage may therefore reflect the toxic effects of the carcinogen rather than the altered genomic expression thought to be responsible for the biochemical abnormalities of some malignant cells.

The hypothesis that tumors are examples of anomalous differentiation and that they may be subject to factors which regulate normal differentiation has received support from in vitro studies[77] of mouse melanoma cells. Low concentrations of bromodeoxyuridine (BUdR) were found to suppress simultaneously both malignancy and the expression of a differentiated function (melanin production) in these cells without markedly affecting their growth. The changes were demonstrable in single cell-derived clones isolated in BUdR and were therefore not due to selection of aberrant cells in the total population. The clones appeared capable of indefinite growth in vitro in BUdR with altered morphology. Inclusion of thymidine with BUdR prevents the analogue-induced alterations and, once initiated, the effects are reversible. BUdR therefore appears to be a useful tool for examining some of the interrelationships between malignancy and cell differentiation.

Regulation of differentiation can occur at several levels. Thus a change in intracellular activity of an enzyme is not necessarily the result of an increase in the rate of synthesis of that enzyme. An important factor in the regulation of thymidine kinase is the stability of its template RNA. The template for this enzyme has been reported to be more stable in certain tumors[78,79] and in regenerating liver[80] than in control liver. This finding has now been extended[81] to the rapidly growing Novikoff rat hepatoma and neonatal rat liver. It is conceivable that template stability is related to the release from the nucleus of mRNA in a "masked"

or protected form, e.g. complexed with specific protein(s). The translation mechanism itself may serve as a protective device for the mRNA: perhaps as long as the ribosomes are rapidly traversing the message, the mRNA is not subject to nuclease activity.

The proteins most often considered as possible genetic regulators are the histones. The remarkable similarities among the F2al histones from human leukemic lymphoblasts, fetal and postnatal calf thymus, bovine lymphosarcoma, and Novikoff hepatoma suggest that these histones may exhibit a general function common to all cells. [82] Small structural differences or different degrees of acetylation and methylation might affect their role as possible gene regulators. The difference in histone methylase activities between adult, fetal, and neoplastic cells for example may be highly significant for maintaining the state of differentiation. Embryonic cells may require intense histone methylation in order to maintain specific histones in a nonfunctional state. This in turn could allow for a time-dependent and orderly selective repression of genes. If a variety of histone methylases exist, their resolution from cells during differentiation followed by specificity studies should prove invaluable in elucidating possible mechanisms involved. Fetal rabbit liver and mouse hepatoma extracts were found [83] to contain higher histone methylase activities than adult mouse and rabbit liver preparations. Embryonic rabbit kidney, brain and spleen tissues were also compared with the corresponding adult tissues. Embryonic tissues had higher histone methylase activity than adult tissues except in the case of spleen. However, embryonic spleen is essentially nonfunctional at this stage of development.

Modifications of transfer RNA's as well as of histones have been suggested to play a role in regulating gene expression. Normal adult rabbit tissues contain inhibitors of the enzymes that methylate tRNA. [84] The inhibitor can be separated into a high molecular weight protein and a low molecular weight component. The larger component has now been shown [85] to be absent from embryonic and tumor tissues, both of which are known to possess high tRNA methylase capacity. Isolated nucleoli of the Novikoff ascites rat tumor have been shown [86] to contain at least two separate methylation activities which utilize S-adenosyl methionine, one acting on ribose groups of nascent preribosomal RNA and one capable of acting on the nucleotide bases of an exogenous nonhomologous tRNA. The tRNA methylase activity and the histology of several transplantable mouse hepatomas have also been examined. [87] Three (H-1, H-4, and H-6) were relatively fast growing. H-1 and H-6 had higher sRNA methylase activity, H-4 had amounts of activity similar to those of liver, and H-3 had variable amounts. sRNA methylase

activity did not seem to be correlated with growth rate but may have been associated with the state of differentiation.

Alterations in rat hepatoma tRNA patterns have been detected[88] by cochromatography of aminoacylated tRNA's from the hepatomas and from normal rat liver. The iso-accepting species of Leu-, Lys-, and Tyr-tRNA from hepatomas 5123D and 3924A appeared identical with those of normal liver. The Ser-, Phe-, and His-tRNA's from hepatoma 3924A also showed no deviation from normalcy. Hepatoma 5123D exhibited two more Ser-tRNA species, one more Phe-tRNA species, and two fewer His-tRNA species than normal rat liver. The new tRNA's were apparently related to the malignancy of the cells and not to their growth rate, since none of the tRNA's examined in regenerating liver differed from those of normal liver.

Investigation of tRNA's in human malignancies has revealed[89] different A/U and G/C ratios in those from spleens of patients with chronic lymphatic leukemia or Hodgkin's disease, as compared with tRNA's from normal human spleens. Of the minor nucleosides, both pseudouridine and 6-methylaminopurine riboside showed a higher mole percent value in the neoplastic tRNA. The occurrence of a third $tRNA^{Phe}$, in addition to the two normally present, has been demonstrated[90] in spleens from patients with various types of lymphomas. These three tRNA's could be charged by using autologous amino acyl synthetases. With the use of amino acyl synthetases from human embryonic liver, several additional tRNA species were identified in the spleens of patients with chronic myelogenous leukemia. The number of species detected depended on the age of the embryo from which the enzymes were derived.

Several other recent studies have provided further evidence of fetal or neonatal biochemical patterns in tumors. High glutathionase (GSH-ase) activity is found in rat hepatomas of different origins and varying growth rates. No activity can be detected in normal adult rat liver, regenerating liver, or livers of tumor-bearing hosts.[91-93] In embryonal and neonatal rat liver, on the other hand, high GSH-ase activity is present.[93] These observations indicate that as a result of malignant transformation, a neonatal biochemical feature is reactivated in rat liver, whereas differentiation during normal development leads to the suppression of this embryonic character. It is not yet known whether activation of GSH-ase during rat liver carcinogenesis is simply a manifestation of the proliferation of primitive cells which cannot differentiate or whether there is a derepression in the parenchymal cells of a latent property which is normally repressed in the adult state. The facts seem more compatible with the second alternative: a very high GSH-ase activity was found in the

Reuber H-139 hepatoma which is a well-differentiated tumor containing glycogen, producing bile, and containing a level of glucose-6-phosphatase activity as high as in normal adult rat liver. Moreover, withdrawal of the carcinogen before the "critical stage" of carcinogenesis led to a substantial decrease, if not a complete loss, of enhanced GSH-ase activity, paralleling roughly the loss of protein-bound carcinogen.

Benzpyrene (BP) hydroxylase is another example of an enzyme which in certain tumors exhibits behavior characteristic of fetal rather than adult tissue. Several transplantable rat hepatomas examined[94] had low or negligible basal levels of BP hydroxylase and low induced levels when compared with normal adult rat liver. The amount of basal activity and the magnitude of induction after treatment with 3-methylcholanthrene were, however, within the range exhibited by fetal and neonatal liver. It is not known whether this phenomenon represents a block in the maturation process or a reversion to the fetal situation. In another group of rat hepatomas, 3-methylcholanthrene-induced hydroxylation of 2-acetylaminofluorene was examined.[95] In normal liver both N-and ring-hydroxylation of 2-AAF are induced, whereas in the tumors only ring-hydroxylation was increased except in the case of hepatoma 5123CTC. This well-differentiated hepatoma showed a 4-fold increase in N-hydroxylating activity which appears to be the first example of N-hydroxylation of 2-AAF by any tumor tissue so far studied.

Most reports concerning hormonal, dietary, and other metabolic regulations of enzyme activity and enzyme synthesis in tumors imply that the regulatory mechanisms in the tumor are absent or impaired, and because of this deficiency ability to respond to regulators is defective. On the contrary, glutamine synthetase has been shown [96] to respond readily to metabolic regulations in some hepatomas while, under the same conditions, the host liver enzyme is unresponsive. Although glutamine synthetase in neonatal rat liver, as well as in the hepatomas, responded more readily to metabolic modulations than that in adult liver, cell division per se does not guarantee responsiveness, because the enzyme in regenerating liver behaved the same as that in normal liver. A deviation from normalcy does not necessarily imply a deletion or deficiency in control mechanisms. Retention of certain features of the regulatory apparatus that are normally deleted in adult liver, or reactivation of normally repressed controls could explain the results equally well.

The only consistent biochemical defect yet found in hepatomas is of the more usual type involving unresponsiveness to normal regulatory molecules. Deletion of the cholesterol feedback system has been demonstrated in all

hepatomas regardless of host species, degree of tumor differentiation, or tumor-inducing agent. The defect has been localized [97] to the enzymatic step responsible for conversion of 3-hydroxy-3-methylglutarate to mevalonate but it has not been determined whether the lesion represents an alteration in the enzyme itself, a derangement in either transcriptional or translational control of the synthesis of the enzyme, or an alteration of an inhibitor of this reaction.

Whatever the defect in cholesterol regulation may be, the available data indicate that it is intimately associated with the malignant state. It may, in fact, precede by months or even years the earliest overt evidence of malignancy. Very small doses of the hepatocarcinogen aflatoxin cause loss of cholesterol feedback control in trout liver long before any morphological change takes place.[98] Feedback deletion in trout liver occurs within one week after feeding a large dose of aflatoxin. It is significant that trout intestine retains the cholesterol regulatory system even after aflatoxin feeding: aflatoxin does not cause intestinal tumors in this species.[98]

There is now evidence that tumors other than hepatomas may also have a defect in cholesterol feedback control. Lymphoid tissue from guinea pig leukemia L_2C synthesizes cholesterol at 4 to 5 times the rate of normal lymphoid tissue and, in contrast to the latter, cholesterol synthesis in the leukemic cells is totally unresponsive to exogenous cholesterol.[98]

Defective regulation of cholesterol biosynthesis is only one of several aberrations of lipid metabolism which have been found in tumors. Incomplete synthesis of the glycolipid carbohydrate chain with simultaneous increase of precursor glycolipids has been demonstrated in several types of tumors. In one such study [99], the glycolipids of three lines of Morris hepatomas were compared with those of neonatal and adult rat liver. The glycolipid patterns of normal adult and neonatal livers were similar and were characterized by the presence of trisialoganglioside and larger proportions of N-acetyl-and N-glycoly-hematosides. Neonatal liver, however, contained a larger quantity of trisialoganglioside and a smaller quantity of monosialoganglioside than did normal adult liver. The patterns of the hepatomas resembled each other but were distinctively different from those of normal adult or neonatal livers. The tumors were characterized by the absence of trisialoganglioside and a high level of disialoganglioside. A relative decrease of the level of hematosides and an increase of neutral glycolipids and ceramide were also observed. Although the total quantity of glycolipids in neonatal liver

expressed on a protein basis was higher than in normal adult liver, the qualitative pattern of glycolipids was quite similar to that of normal liver. It is of great interest that irrespective of the difference in the absolute quantity of glycolipids, the differences in qualitative patterns of glycolipids between normal and rapidly growing neonatal liver were similar and that they differed greatly from that of the tumor tissue although some of the tumor tissue grew more slowly than did neonatal tissue. Thus the qualitative glycolipid pattern of these tissues is not simply related to the rapidity of cell growth but is more closely related to the intricate quality of the membrane. Although the altered pattern of glycolipids in the three tumors was similar, the accumulated disialoganglioside was correlated with the degree of malignancy.

In a related study,[100] the glycolipids of normal human and hamster fibroblasts were analyzed and compared with those of virus-transformed cells of the same types. The concentration per mg protein of certain glycolipids appeared to increase in the normal cells at high cell densities. After transformation by SV40 or polyoma virus, the glycolipids which responded to cell confluence either disappeared or were greatly reduced in concentration, suggesting that these membrane components may be involved in the contact inhibition of normal cell division which is lost in malignant cells.

Exposure of cells to neuraminidase permits an evaluation of the contribution of the carboxyl of sialic acid in the net surface charge. The increased amount of sialic acid which has been reported to characterize the surface of the malignant cell was not observed [101] in a comparison of normal lymphoid cells and macrophages and leukemic cells from three strains of mice. The malignant cells did not generally show increased surface charge density as compared with their normal counterparts. These results may not, however, be unexpected in light of the fact that neither of the normal non-malignant cells examined exists in cell associations but rather only as free entities. Similar results were in fact obtained in a comparison [102] of electrophoretic mobilities of human peripheral lymphocytes, peripheral malignant cells from patients with acute and chronic leukemia, cultured "normal" lymphoblasts, and cultured cells from Burkitt's tumors. No consistent differences were observed between the electrophoretic mobilities of the isolated but otherwise untreated cells. Both normal and malignant cells showed similar reductions in net surface negativity following treatment with neuraminidase, thus contradicting the generality that ionized sialic acid moieties contribute more to the S-potentials at the surface of malignant cells than to those of their normal counterparts. In contrast to the re-

sults with neuraminidase, incubation of the cells with ribonuclease A caused the net surface negativity of the normal peripheral lymphocytes and cultured "normal" lymphoblasts to be significantly reduced, whereas the surface negativity of the peripheral leukemic cells and the cultured cells from Burkitt lymphomas remained relatively unaffected. This confirms previous reports of the presence of RNA as a structural component of the cell periphery and supports the concept [103] that this peripheral RNA may be involved in cell recognition processes such as contact inhibition.

An impaired response to contact inhibition of cell division is one of the most consistent behavioral features of cancer cells in vitro. The behavioral change appears to be associated with an alteration in the chemical configuration of the cell surface which renders the malignant cells subject to agglutination by concanavalin A(conA). Normal cells will normally bind this phytoagglutinin only during mitosis [104] but can be rendered susceptible during other phases of the cell cycle by treatment with trypsin.[105] The observation of a membrane alteration occurring during a short period of the normal cell cycle has led to the suggestion [104] that this surface change is a trigger for the processes in the cell cycle leading to the next mitosis. By affecting activities such as RNA or protein synthesis, transport, or concomitant nuclear membrane changes, the surface alteration might be responsible for essential steps in the subsequent S,G-2, or M phases of the cell cycle. While the surface change responsible for susceptibility to conA is undoubtedly only one of many cellular mitotic alterations, it apparently plays a key role in regulating cell division. This conclusion is supported by the results of experiments in which mouse fibroblasts transformed by polyoma virus were treated with monovalent conA. The monovalent molecule, obtained by splitting with trypsin, can bind to transformed cells but does not agglutinate or kill them. When the available binding sites on the transformed fibroblasts had bound the split conA, the cells were again subject to contact inhibition and grew like normal cells.[106] This process could be reversed by addition of either α-methyl-glucopyranoside or α-methyl-mannopyranoside, the hapten inhibitors of conA. A similar correlation between masking of conA binding sites and susceptibility to contact inhibition has previously been observed in hamster embryo cells. Clones of polyoma virus-transformed hamster embryo cells were shown to produce a high frequency of variants in which the properties characteristic of transformed cells, e.g. loss of contact inhibition, had reverted to the normal state.[107] This reversion was not associated with loss of the viral genome. The "normal" revertants were subsequently shown to have undergone a loss of agglutinability by conA; loss of agglutinability was not complete in all cases. Susceptibility

to agglutination by conA could be restored by treating the revertants with trypsin.[108] It has been estimated that the number of exposed binding sites for conA on normal cells is 4 to 8 x $10^3/\mu^2$ and 23 to 58 x $10^3/\mu^2$ for the transformed cells.[109] It appears, therefore, that a threshold of exposed sites must be exceeded before the cells express the in vitro properties of transformation.[108]

HOST-TUMOR INTERACTIONS

The etiologic role of the Epstein-Barr virus (EBV) in human malignancy continues to be assessed. Recently DNA has been isolated from partially purified EBV, making it possible to determine that viral nucleic acid is present in tumor cells. DNA hybridization experiments have provided direct evidence that EBV-DNA is present in biopsies of Burkitt's lymphoma (BL) and anaplastic nasopharyngeal carcinoma (NPC).[110] Up to 26 genome equivalents were estimated to be present per cell. The number of viral genome equivalents were of the same order in different tumors of given BL patients biopsied at different times suggesting a stable virus host relationship in individual patients. Serological tests on 96 African BL patients also revealed antibody activity against EBV-associated membrane antigens (MA) and EBV capsid antigens (VCA) in different disease stages indicating that patients with BL are subject to continued antigenic stimulation from EBV antigens.[111] Antibodies against EBV-induced MA and not EB-VCA or EBV-induced early antigen are apparently responsible for neutralization of viral infectivity.[112] This suggests that these antigens are also expressed on the infectious viral particle. Since most herpes viruses derive their envelope from a membrane of the infected cell, it is possible that virus-induced MA becomes part of the envelope of the mature virus particle. Preliminary studies show that when enveloped virus particles are separated from non-enveloped virus particles by density-gradient centrifugation, only enveloped particles are infectious. The expression of the viral genome in BL tumor cells in situ appears to be under more restrictive control than in cultured cells since EBV antigen-producing cells may often emerge rapidly in culture even though the biopsy cell suspensions rarely reveal such cells.[113]

Cross reactivity, if not identity, has been demonstrated between the EBV-associated MA complexes present on lymphoblastoid cell lines, whether they are derived from BL, NPC or infectious mononucleosis (IM). This suggests an association with a related herpes virus.[114] Seroepidemiologic studies reveal that the herpes-like virus (HLV) associated with BL, NPC and IM is also associated with sarcoidosis. All sarcoidosis patients studied[115] had antibody to HLV, antibody titers being higher in the chronic disease group and those who had apparently recovered than in those with early subacute disease. In sarcoidosis and possibly in BL and NPC, the high antibody titers may, at least in part,

reflect not the intensity of the ongoing infection, but an enhanced humoral-antibody response. In a study[116] of over 100 Hodgkin's disease patients, only those patients who manifested lymphocyte depletion--the sarcomatous form--had high titers to EBV. In another population of 57 patients with Hodgkin's disease, lacking examples of lymphocyte depletion, the incidence of antibody titer to EBV, herpes simplex and cytomegalovirus did not differ from an age-matched control population.[117] These studies do not support the concept that the EBV replicates opportunistically against a background of any lymphoproliferative disease, making the association of EBV with human malignancy all the more significant.

Because of the association of EBV with IM as well as with the malignant processes, the relationship between IM and malignancy is of interest. There has been speculation that IM might be an abortive form of acute lymphatic leukemia (ALL). Data from a study[118] of 4 patients with concurrent IM and ALL do not support this hypothesis, however. In one of these patients IM preceded ALL by 3 months, and in 3 other patients occurred at various times during hematologic remission. The occurrence of IM did not alter the clinical expression of ALL. It appears that IM is merely an intercurrent illness which is not etiologically related to ALL. Other studies, however, suggest that IM could well be viewed as a model of a neoplastic or preneoplastic process in the human which is almost invariably successfully host regulated. Transplantation experiments[119] have shown that cells isolated in continuous suspension culture from the peripheral blood of a young adult with IM, exhibited malignant behavior by forming immunoglobulin-synthesizing, invasive, metastatic tumor upon injection into neonatal Syrian hamsters. This may represent release of IM cells from the normally exercised restraints upon expression of their malignant potential. The relationship of EBV and IM to malignancy is further complicated by the fact that a cell line isolated from "normal" blood also has produced serially transplantable tumors in ALS-treated newborn hamsters. Observations such as these may ultimately compel revision of existing concepts concerning the distinction between "normalcy" and "malignancy." Normalcy and malignancy of cell populations may come in significant measure to be regarded as relative to the integrity of host control mechanisms that normally prevent the release of cells from the usual pathways of maturation and differentiation.

Antigenic studies of mouse tumors support the concept that cancer development involves tissue dedifferentiation, i. e., a regression to an ontogenetically more primitive level of maturation. The reappearance and the persistence of embryonal antigens in malignant cells of the mouse appear to be universal phenomena.[120] These antigens were not demonstrated in any normal adult tissue except skin but were demonstrated in 18 different mouse strains in 12 diverse tissues following induction by

oncogenic viruses in 21 instances (Bittner, Gross, Rauscher and Friend), and by chemical carcinogens in 20 instances, in 2 instances following X-irradiation and in 29 instances after spontaneous malignant transformation. While these antigens are likely to become useful in both diagnosis and therapy, more precise data are required before their existence can be fully exploited. A preliminary examination[121] of the role of fetal antigen in transplantation resistance indicates that irradiated hamster and mouse fetal tissues contain antigens which evoke immunity to SV40-induced tumors in syngeneic hamsters. The complexity of the situation is illustrated by the fact that in a similar study involving the mouse mammary tumor system, no protective effect was demonstrated following immunization with fetal tissue.[122]

Neutralization of mouse mammary tumor virus by serum from women with breast cancer has been demonstrated.[123] The presence of neutralizing antibodies in sera from human breast cancer patients and their absence from normal human sera may indicate a viral etiology for the human disease.

Genetic derepression of thymus leukemia antigen Tla genes in mouse leukemia indicates that this region of chromosome IX may in some way be involved in a type of leukemogenesis in the mouse. The TL.4 antigen is leukemia-specific and is never found on any non-leukemic cells. It has now been established that the TL.4 determinant is a cellular gene and the TL.4 gene belongs to the Tla locus, which is linked to the D end of H-2.[124] By immunoselection, an H-2 loss variant line, lacking $H\text{-}2^a$ antigens, was obtained from an $H\text{-}2^b$ /$H\text{-}2^a$ transplanted leukemia.[125] The TL phenotype of the unselected line was TL. 1,2,3,4 and that of the variant TL.1,2,4, indicating that the deletion involved in variant formation extended beyond H-2 to include Tla, despite absence of selection against cells carrying TL antigens. The continued malignancy of the variant despite the functional deletion of one Tla locus, tends to exclude the hypothesis that two aberrant Tla loci are necessary for TL malignancy.

Using visually labeled antibody, G(Gross) and H-2 antigens have been detected on G leukemia virus-infected cells in discrete circumscribed areas.[126] Neither antigen was present on free G virions or on virions in the process of budding from the cell surface. Thus G cell-surface antigen identified by C57BL mouse cytotoxic antiserum is not a constituent of the viral envelope, which accounts for the poor virus-neutralizing capacity of such antibody. NZB mice, in contrast to C57BL and other strains, form antibody to the envelope antigen of Gross virus. A failure or breaking of tolerance is implied in this case, and this may have a relationship to the nephritis which accompanies the autoimmune syndrome characteristic of NZB mice.

Human neoplasms are similar to experimentally induced animal neoplasms in that they generally contain specific tumor antigens. Tumor specific antigens are capable of stimulating both humoral and cell mediated responses on the part of the tumor-bearing host. Human malignant melanoma[127] and sarcoma[128] antibodies have been demonstrated by immunofluorescence. Cellular immunity to antigens which are common to human colonic carcinomas and fetal tissue has been demonstrated by the colony inhibition technique.[129] Lymphocytes from patients with colonic carcinoma can inhibit colony formation of plated cells not only from colonic carcinoma but also from fetal gut and liver epithelium. Sera from patients with colonic carcinomas can block tumor-cell destruction by the same patient's immune lymphocytes. Similar immune reactions mediated by peripheral blood lymphocytes have been demonstrated against tumor-associated antigens of human neuroblastoma.[130] Colony inhibition of neuroblastoma cells is observed not only by autochthonous lymphocytes but also by lymphocytes from some parents and siblings of neuroblastoma patients. Sera from patients with progressively growing tumors can block lymphocyte-mediated tumor-cell inhibition in vitro, whereas sera from patients with regressing tumors cannot. This blocking activity is attributed to the presence of enhancing antibodies. Serum blocking factors have also been revealed in radiation-induced canine chimeras[131] and in mice made tolerant as newborns by inoculation of allogeneic cells.[132] In the murine Moloney sarcoma system, when regressor serum is mixed with progressor serum, the blocking effect of the latter is decreased.[133] Procedures which might decrease the amount of blocking antibodies, e.g. administration of regressor sera or sera from healthy relatives, could, if successful, be expected to be therapeutically useful.

The role of cell-mediated immunity in host resistance to oncogenic processes and the role of thymosin (a lymphocytopoietic factor from thymus) in the enhancement of cell-mediated immunity in thymectomized neonatal mice suggested that the administration of thymosin to mice might augment their resistance to infection with an oncogenic virus.[134] Pretreatment of CBA/WL mice with thymosin significantly accelerated the rate of development of resistance to Moloney sarcoma virus-induced tumors.[135] The data suggest a humoral role for the thymus in the development of tumor immunity and that normal development of cell-mediated immunological competence can be accelerated through the use of thymus-derived extracts.

The degree of histocompatability of three women with trophoblastic tumors evaluated with respect to 14 of their children does not support the hypothesis[136] that the survival and dissemination of postgestational choriocarcinoma is dependent upon a high degree of histocompatability between the malignant graft and the maternal host.[137] In one-way mixed leukocyte cultures, cells from all 14 of the children stimulated their mother's lymphocytes indicating the presence of antigenic differences. However, leukocyte

serotyping showed that cells from 6 of these children possessed only antigens that were present on their mothers' leukocytes. Incompatability did not protect against dissemination since two of the 3 patients had metastatic tumor. A circulating cytotoxic antibody was demonstrated in the patient with locally invasive disease and this antibody reacted with lymphocytes from the patient's husband and 3 of her 6 children.

Recent studies of the Australia antigen AU(1), a hepatitis-associated antigen, have contributed to revised concepts of viral hepatitis. Preliminary studies demonstrating the replication of AU(1) in the serum of an African green monkey 24 hours after it had been inoculated with antigen isolated from man, support the hypothesis that AU(1) is a virus.[138] It is likely that there are at least two different viruses that can cause acute viral hepatitis. One of these viruses [AU(1) virus] can be detected by the presence of AU(1) and the other cannot. The term "serum hepatitis" now appears misleading and perpetuates the false concept that a particular hepatitis virus can be identified by its mode of transmission. AU(1) has been found in high frequency in patients with leukemia (7.0% of 688 patients) and Hodgkin's disease (6.3% of 80 patients); its frequency in the normal U. S. population is 0.1%.[139] The antigen in these patients is associated with blood transfusions but may occur without transfusion. Persistent AU(1) in leukemic patients probably represents chronic anicteric hepatitis. Although transfusion resulted in an increased frequency of AU(1) in patients with chronic and acute lymphocytic leukemia compared with nonleukemic patients, this was not true for patients with chronic and acute myelogenous leukemia. This favors the explanation that persistent infection results from immune defects in lymphocytic leukemia.

Phytohemagglutinin (PHA)-induced transformation of lymphocytes is decreased in certain lymphoproliferative disorders, particularly in chronic lymphocytic leukemia (CLL). A wide range of responsiveness of peripheral blood lymphocytes to PHA was found in 13 cases of lymphosarcoma (Stage IV), with a median value for the group lower than that of controls.[140] Lymphocyte transformation correlated with histologic type. The most marked impairment of transformation generally occurred in patients with small cell lymphosarcoma, in patients with brief duration of disease and in older age groups. In this group, there was little correlation between peripheral blood lymphocyte level and percentage of transformation. In 6 patients with reticulum cell sarcoma (Stage IV), transformation was markedly impaired. In 17 patients with Hodgkin's disease, impaired transformation was related to stage of disease and peripheral lymphocyte levels. In other studies the finding of similar metabolic and biosynthetic abnormalities in lymphocytes from patients with diabetes mellitus and CLL may provide an additional explanation for the failure of patients with both disorders to resist infection.[141] Lymphocytes

demonstrated depressed glucose utilization via the hexose monophosphate shunt and decreased incorporation of radioactive thymidine, particularly under the influence of PHA. A correlative, semi-quantitative relationship was present between the amount of glucose metabolized through the direct oxidative pathway, and the synthesis of DNA. The data imply that normal antigenic recognition and the satisfactory initiation of the immune response, both requiring adequate DNA synthesis, may have a metabolic basis.

A factor that is present in the serum and urine of animals and humans and which can stimulate the formation of granulocytic colonies by normal mouse-bone-marrow cells in vitro, is altered in patients with leukemia.[142] Urine from eight patients with chronic granulocytic leukemia had high levels of colony-growth-stimulating activity (CSA). Six of seven patients with acute stem-cell or acute lymphatic leukemia also had high levels, whereas 9 of 15 patients with untreated acute granulocytic leukemia (AGL) had low or absent levels. In AGL, levels may rise during treatment, often become normal to very high with remission, only to fall again to subnormal levels with relapse. Immature granulocytes from untreated patients with AGL will not stimulate normal human-bone-marrow colony growth. This suggests that normal mature granulocytes may be the major source of the factor responsible for CSA and that the low levels of AGL may reflect the absence of mature granulocytes in these patients. A combined neoplastic, accumulative hypothesis might be envisioned in AGL where cells have not lost the ability to mature normally but merely lack the stimulus to do so.

It has long been known that tumor cells are capable of eliciting neovascularization. A soluble factor has now been isolated from human (hepatoblastoma, neuroblastoma and Wilm's tumor) and animal (Walker 256 carcinoma and mouse B16 melanoma) neoplasms which is mitogenic to capillary endothelial cells.[143] Neovascularizing activity was demonstrated using the rat dorsal air sac technique. This tumor-angiogenesis factor, contains approximately 25% RNA, 10% protein, 50% carbohydrate, the remainder being presumably lipoidal material.

A unifying concept has been proposed for the etiology of multiple endocrine adenomatosis and the inappropriate secretion of peptide hormones by nonendocrine tumors.[144] It is proposed that the peptide-secreting endocrine tissues are related to the nervous system not only by their coordination of bodily function but also by their development from primitive multipotential endocrine cells derived from neural ectoderm. These neuroendocrine cells migrate into the primitive alimentary tract mucosa and are carried with the buds of the various developing endocrine glands to their final locations, where they mature into endocrine cells of the anterior pituitary, thyroid, parathyroid, islets of Langerhans, ultimo-

branchial body and thymus. Neuroendocrine cells in the hypothalamus produce the posterior pituitary hormones, and related cells (argentaffin cells) are present throughout the alimentary tract mucosa and in the various derivatives of the gut--bronchi, salivary glands, pancreatic and biliary ducts, bladder and genitourinary system. Related cells can be found in the adrenal medulla, sympathetic ganglia, paraganglia and glomus or chemoreceptor system. These widely scattered neuroendocrine cells have the capacity to develop into a wide variety of peptide-producing endocrine tumors. If this hypothesis is correct, the syndrome of multiple endocrine adenomatosis may simply be a dysplasia of neural ectoderm.

A 20-year study[145] involving 435 range beef cattle has demonstrated an association between nutrition and eye cancer. Animals on a high intake diet had a higher incidence of ocular squamous-cell carcinoma than those on a low intake diet. More animals with more advanced lesions were observed at earlier ages in the higher feeding levels than in medium or low levels. In another study, the highest incidence of spontaneous chromophobe adenomas of the anterior pituitary gland of male COBS strain rats was also found to be associated with the dietary group having the highest intake of food.[146] Under restricted conditions tumor prevalence was directly related to the level of the protein component in the diet. Restriction in both caloric intake and protein intake depressed the incidence to the greatest extent. Body weight of the rat early in life correlated directly with tumor prevalence of later life. Specific dietary factors have been found to influence the development of several other types of tumors. Sprague-Dawley rats receiving zinc-deficient diets showed a marked reduction of Walker 256 carcinosarcoma growth and this was accompanied by a striking increase in survival.[147] The requirement for zinc by growing tumors suggests a possible explanation for the observed decreased levels of zinc in sera or cells from human cancer patients. In mammary tumor virus-infected C3H mice the transformation of normal mammary tissues to hyperplastic alveolar nodules was inhibited by diets deficient in phenylalanine.[148] Phenylalanine deficiency, aided by partial starvation, appears to be responsible for the inhibition which may act via a disturbed normal milieu as evidenced by poor mammary gland development and presence of abnormal ovaries. Feeding a riboflavin deficient diet to 3-4 week old ICR female mice tended to enhance 7,12-dimethylbenzanthracene-induced skin carcinogenesis,[149] but vitamin E deficiency did not accelerate the induction or growth of tumors by a low level (0.005%) of N-2-fluorenylacetamide in rats.[150]

A positive association is inferred among abnormal lipoprotein biochemistry, familial relationship, and the possibility of developing cancer.[151] Sera of men, women, and children with cancer have unusually low values of the high-density lipoprotein component, HDL_2. The presence of cancer also seems to increase greatly the levels of very-low-density lipoproteins, especially in men. Ab-

normally low values for HDL_2 in normal subjects were usually a characteristic of the individual, and these were associated with a pronounced positive history of cancer in close blood relatives. Four normal subjects (2 men and 2 women) with positive family histories of cancer and very low values for HDL_2, who themselves had no evidence of disease when the samples were taken, have since developed cancer. The two men also had markedly elevated values for very-low-density lipoproteins.

EPIDEMIOLOGY

An international collaborative study of breast cancer and reproductive history carried out in seven widely scattered cities of the world did not reveal consistent differences in duration of lactation between breast cancer patients and controls, once the fact that breast cancer patients have fewer pregnancies had been allowed for.[152] In all areas studied, breast cancer risk was strongly correlated with age at first pregnancy.[153] Women having their first child when under 18 years of age had only 1/3 the breast cancer risk of those whose first birth was delayed until the age of 35 years or more. Subsequent pregnancies even if they occurred at an early age, showed very little, if any protective effect. The reduced risk of breast cancer in women having their first child at an early age explains the previously observed inverse relationship between total parity and breast cancer risk, since women having their first birth early tend to become ultimately of high parity. The data suggest that late first pregnancies may actually increase breast cancer risk. Women who had their first delivery after the age of 35 years had risks approximately 20% higher than those who were nulliparous and subsequent births appeared to be associated with an additional increase in risk. Increased weight in women over 50 years of age is also associated with increased breast cancer risk.[154] The restriction to two different age groups of the two major positive findings of the study (association of risk with late first pregnancy and with weight) suggests that there may be two periods of life when a woman's risk of breast tumor induction is particularly high. The decade or so commencing at puberty may be one of these periods, and alterations of risk at this time may be associated with the first pregnancy. The decade or so surrounding the menopause may be the second high risk period and alteration in risk during this period may be associated with weight.

The finding of differences in histologic pattern between young adult (15-34 years) and elderly (50+ years) patients with Hodgkin's disease tends to support the multiple-disease hypothesis.[155] Clinical factors and 23 histologic features were entered as additive linear terms into a discriminant function analysis. The feature with the strongest discriminating power was the presence of eosinophils, followed by atypical mitosis, areas of preserved architecture, extent of disease, Reed-Sternberg (R-S) cells, and extent of fibrosis. High scores for extent of disease

and atypical mitosis were characteristic of the older age category. High scores were observed among young adults for R-S cells, eosinophils, and extent of fibrosis. All the latter features are components of the "nodular sclerosis" type of Hodgkin's disease. These observations suggest that "Hodgkin's Disease" encompasses at least two etiologic entities -- one with a peak incidence in young adults and the other with a peak incidence in the elderly. Several distinctive and pathologic features characterize Hodgkin's disease in patients in the first decade of life.[156] A study of 35 children with this disease revealed a striking predominance of males (91%). There are very few substantiated cases of Hodgkin's disease occurring at ages less than 3 years. In 30 of 31 classifiable cases, the histologic sections showed either nodular sclerosing Hodgkin's disease or Hodgkin's disease with lymphocytic predominance. In 20 patients R-S cells were difficult to find. The great majority of cases, regardless of classification, were characterized by a striking preponderance of mature lymphocytes, suggesting that a particular host response characterizes the disease of the young.

A study[157] investigating the role of lymphoid tissue in neoplasia has shown no evidence that prior appendectomy or tonsillectomy affects the subsequent risk of cancer. This relationship was studied in a series of 919 autopsies and in an interview series of 461 cancer patients and 223 controls. However, the relatively small sample sizes of patients with particular neoplasms do not permit a conclusive statement concerning the absence of a relative risk.

A 26-year survey[158] of cancer mortality in 21,447 diabetics revealed a significantly reduced cancer risk for male diabetics. Deficiencies in deaths from cancer of the respiratory tract and rectum were partly responsible for this difference. The decreased risk for the male diabetics was attributed to two factors: markedly excessive risk of death from other causes and an over-representation of Jews among the patients (Jewish men have a diminished lung cancer risk). There was a significantly decreased risk of cancer of the uterus, both of the body and of the cervix, in women with diabetes. Increased risk of pancreatic cancer in both sexes was confirmed. It was suggested that an investigation of the carcinogenic potentialities of exogenous animal insulins may clarify the mechanisms involved in the association of diabetes and pancreatic cancer.

An analysis[159] of the data from the Tri-State Leukemia study did not show differences among children between cases and controls in religious and ethnic background, nor in any demographic variable. Among the more than 1200 adult cases and 1200 adult controls, however, Jews, Russians and Poles all had significantly elevated risks. For Russian-Jews the risk was over five times that of non-Jews born in the United States. On the other hand,

the finding that the degree of their excess risk is not as great when compared with populations culturally more similar to themselves, suggests the importance of either a social or a genetic trait, or both. The relationship observed for Jews did not vary by histologic type of cancer and no differences in irradiation experience among religious or ethnic groups were discovered.

In East and Southeast Asia, nasopharyngeal carcinoma (NPC) occurs with extraordinarily high frequencies, especially among the Chinese. A study[160] was undertaken of Singapore Chinese with special reference to migrant status and specific dialect group in order to evaluate the respective roles of genetic and environmental factors in the genesis of this neoplasm. The number of China-born cases was significantly lower than expected when compared with Singapore-born Chinese but not when compared with controls. While NPC was common in all segments of the immigrant and local-born Chinese population of Singapore, the relative risk was lower for those derived from the Province of Fukien and higher for those belonging to the Cantonese dialect group. The high relative risk among Cantonese and the low relative risk in Fukien (Hokkiens) are less marked among Singapore-born Chinese than among the China born. A higher risk for the Hainanese was also observed among the China-born but not the local-born. The highly significant dialect group differences observed, similar to the observations reported in Hong Kong, probably represent real differences in risk unrelated to any local geographic factor.

Criteria were sought[161] that would assist the operating surgeon in differentiating metastasizing from nonmetastasizing colorectal carcinomas. Characteristics associated with an increased probability of metastasis to mesenteric lymph nodes included the past or present association of skin cancers, the existence of metastases outside the field of resection, and the characteristics of the cancer margins (greater stromal and vascular infiltration by cancer and less inflammatory cell infiltration). Factors associated with a decreased likelihood of metastasis to lymph nodes included favorable characteristics of the cancer margins ("pushing" or circumscribed margin and denser infiltration of inflammatory cells), deep local extension through the bowel wall into mesenteric fat and adjacent organs, and absence of distant metastases. The nonmetastasizing variants were found among tumors of all sizes and histologic grades.

Trends in incidence and mortality rates for cancer of the gastrointestinal tract have been analyzed from the data of the Connecticut Tumor Registry for the 30-year period 1935-1964.[162] While cancer of the rectum makes up about one-fifth of all malignancies of the digestive system there was little variation in incidence over time or between sexes. The proportion classified as colonic cancer, however, has increased over this period. By 1960-64 cancer of the colon made up the largest part of total

digestive system malignancies, increasing from 21 to 33% for men and from 31 to 46% for women. When a breakdown by segments of the colon is made, sigmoid colon had the highest incidence rate and has increased from 8 to 12 per 100,000 men and from 8 to 11 per 100,000 women. The rate for ascending colon has shown the greatest amount of change with an increase from 4 to 8 per 100,000 men and from 5 to 10 per 100,000 women. Transverse colon also has increased from 3 to 5 per 100,000, and the descending colon with the lowest incidence has increased from little over 1 to nearly 3 per 100,000. Incidence of cancer of the small intestine, liver, gall bladder and esophagus has changed little over the 30-year period. Cancer of the pancreas has increased in men from 7 per 100,000 to 10 per 100,000. The proportion classified as stomach cancer decreased steadily from 37 to 31% for men and from 28 to 14% for women.

The relative annual frequencies of cancers seen among Missouri residents treated at the Ellis Fischel State Cancer Hospital have changed during the period 1940 to 1969.[163] Consistent trends since 1940 show a decrement in the frequency of both skin and lip cancer. Cancers of the respiratory system, blood-forming organs, urinary tract and lower digestive tract have shown an increase. Primary non-specified (dedifferentiated) cancers were also increased. The frequency of cancers of other sites has stayed remarkably constant. No conclusions regarding possible causes of these trends are possible, and a variety of underlying variables could account for these observations. During this same time period the number of cancers seen among Missouri farmers has decreased, and of those seen among smokers and those exposed to air pollution, has increased. Simultaneously, the population has shifted in predominance from rural to urban.

Analyses of the association between obstetric x-rays and childhood malignancies support the gene-selection theory[164] of cancer: cancers caused in this way are due to the propagation of one cell whose controlling gene has experienced a small but irreversible change at the moment of exposure to x-rays; prenatal exposure to x-rays exerts a carcinogenic effect during the first 10 years of postnatal life,[165] directly related to fetal dose.[166] The risk of cancer was greatest when exposure was during the first trimester. The radiogenic cases were evenly divided among 10 diagnostic types of cancer. Lymphatic and stem-cell leukemias had latent periods of 3-5 years; neuroblastoma deaths peaked at 0-2 years and Wilms' tumor at 1-3 years; some cases of lymphoma had latent periods of 7-10 years, and some cases of myeloid and monocytic leukemias were not clinically apparent until after 6 years of age. According to the gene-selection theory there is no fundamental difference between childhood cancers and adult cancers, between benign and malignant tumors, between sarcomas and carcinomas or between radiation cancer and spontaneous cancers. The attractiveness of this "one-hit" theory is that it allows very simple mechanisms to account for the

different types of radiation cancers observed, for various associations between hemopoietic neoplasms and infections and for the way in which cancer prevalence varies with age.

A retrospective investigation[167] of 350 lung cancer patients revealed a lower relative risk of lung cancer (squamous and oat cell types) for individuals who had smoked filter cigarettes for at least 10 years after switching from nonfilter cigarettes than for those who continued to smoke nonfiltered cigarettes. The relative risk for lung cancer among ex-smokers continues to be high for at least three years after cessation of smoking. Thirteen years after an individual has stopped smoking, the relative risk appears to be close to that of individuals who never smoked. Cigarette smoking increases the risk of neoplasms of the lower urinary tract (renal pelvis, ureter, bladder and urethra).[168] Interviews with 470 patients with transitional or squamous cell carcinoma of the lower urinary tract (more than 90% of whom had a bladder tumor) and 500 controls showed that among men, cigarette smokers had a relative risk of bladder cancer of 1.89 as compared with nonsmokers, and about 39% of the cases are related to smoking. This amounts to 16.4 cases per year per 100,000 men 20 years of age and over. Among women 20 years of age and over, the respective figures are 2.00, 29 per cent and 3.9 per year per 100,000. For both sexes risk was increased among those who smoked heavily and those who inhaled. None of the excess risk of bladder cancer associated with cigarette smoking is explained by indirect association with occupational experience. No significant risk is associated with pipe or cigar smoking. The data also suggest that incidence rates may increase during the next decade or so, especially among women.

Abnormal tryptophan metabolites may play a role in heterotopic recurrences of bladder tumors. Followup studies[169] disclosed that all of 18 patients with abnormal tryptophan metabolism had one or more recurrences within 5 years, however, among 20 patients with normal tryptophan metabolism, 8 patients had no recurrences within 5 years, and 12 patients had recurrences within 5 years. The data suggest that it may be possible to retard the development of recurrences by correction of tryptophan metabolism with the administration of vitamin B_6.

Abnormal tryptophan metabolism observed in a variety of diseases including cancer may be the result of increased stress resulting in elevated corticosteroid production and increase in tryptophan pyrrolase activity. This suggests that the degree of stress is related to the extent of disease, and therefore, patients with more extensive disease would be expected to exhibit greater alterations in tryptophan metabolism. Recent experiments[170] support this suggestion. In 2 series of cancer patients, differing by sex and by organ site of disease, i. e., female breast and male bladder, higher mean post-tryptophan levels of urinary metabolites were observed in the category characterized by more exten-

sive disease involvement. In the breast cancer series, both the abnormal metabolism of tryptophan and the advanced state of the disease may be a reflection of endocrine imbalances. In another study,[171] the abnormal tryptophan metabolism observed in breast cancer patients after a loading dose of tryptophan persisted after the administration of L-kynurenine and pyridoxine. No correlation was found between levels of tryptophan metabolites and 17-hydroxycorticoid excretion; however, the urinary excretion of etiocholanolone was significantly lower in breast cancer patients with abnormal tryptophan metabolism, suggesting a mechanism other than stress-induced tryptophan pyrrolase induction. A correlation was not found in the "free interval" between initial mastectomy or recurrence and abnormal tryptophan metabolism.

Epidemiological studies[172] of the frequency of Pseudomonas sp. in stools and throats of 87 patients with leukemia revealed that 25% of the patients were already carriers on admission to the hospital and a total of 54% of the patients became carriers during hospitalization. Patients with leukemia who were carriers had a higher frequency of Pseudomonas sp. infections than those who were noncarriers. In the majority of infections occurring in carriers, the same pyocine type of Pseudomonas sp. was found simultaneously in the stool or throat and at the site of infection. The weekly examination of stools for the presence of infection, especially during the first month of hospitalization, would help to identify those patients having a higher risk of developing Pseudomonas sp. infection. The prophylactic administration of oral non-absorbable antipseudomonal antibiotics would probably reduce the carrier rate and thus might reduce the incidence of infection. Epidemiologic analysis[173] of a 19-case hospital-associated outbreak of Pneumocystis carinii pneumonia showed that the more intensive chemotherapeutic protocols caused the outbreak by activating inapparent infection commonly associated with neoplastic disease. Pulmonary needle aspiration proved to be a safe and rapid mode of diagnosis in these children and should be used when the diagnosis of P. carinii is first suspected.

Epidemiologic evidence suggests the implication of a venereally transmitted factor in the etiology of cervical carcinoma. Antibodies to the genital or type 2 herpesvirus have been found[174] among 83% of women with invasive carcinoma, but in only 33% of women with carcinoma in situ (CIS). Antibodies were found in 22 and 9% of control groups of women from lower and higher socioeconomic classes, respectively. Analysis of the occurrence of antibodies by age among women with CIS and invasive carcinoma suggests that the acquisition of antibodies precedes rather than follows the development of neoplastic change. Not all cervical neoplasms carried the same probability of being associated with antibodies since patients with adenocarcinoma and small cell type squamous carcinoma failed to show the same occurrence of antibodies as was observed with keratinizing and large cell nonkeratinizing squamous carcinoma. No evidence for increased sus-

ceptibility to virus following neoplastic change could be demonstrated. In fact, the age distribution of antibodies among patients with CIS and invasive carcinoma could be construed as evidence for possible multiple etiologies of the cancer. One type may be preceded by CIS in which the virus acts as a carcinogen or co-carcinogen, while in another form of the lesion, the virus may play no role.

Several ways have been described for evaluating the association between antigens in a system and the antibody composition of a panel of sera from the pattern of reaction of these sera with a panel of cells.[175] Two simple mathematical models of the reaction process were investigated: an all or none Boolean model and a linear model which involves a continuous strength of reaction. Data were generated to simulate random immunization under the Boolean model. Two techniques based on the linear model were used on these data: the factor analytic technique of Terasaki and the least squares technique of Elston. Neither algorithm was uniformly superior to the other, and faced with real data, both may be used for the different light they shed. Another mathematical model[176] developed in order to examine the relationship between the local spread of uterine cancer and the movements of the constituent cells of the tumor predicts that when the visible tumor is still quite small the extent of invisible spread is relatively quite large. Examination of clinical data confirms this prediction.

Statistical models have been developed to try to explain the dose-dependence of both the means and variances of survival times of mice receiving ascites tumor cells.[177] The factor found to be potentially explanatory is the variation in host doubling time. The model that took lognormal host doubling time as the sole source of variation was found to work reasonably well in describing the observed data and was used to obtain estimates of the lethal cell number and mean and variance of host doubling time. The model that incorporated both the variations in host doubling time and the number of clonegenic cells did not improve the fit of the model to the data but did provide an estimate of the cell take rate.

The Mantel regression approach was used to test space-time clustering (address and time of diagnosis) among 149 children with leukemia under the age of 15 years, diagnosed in San Francisco during the 20-year period 1946-65.[178] Separate analyses were performed at ages 0 to 14 and 2 to 14 years. Neither of the age groupings showed statistically significant clustering for 2-month intervals. The only analysis of this type yielding a p value less than 0.05 was for ages 2 to 14 years and with 12-month intervals ($p=0.024$). A comparison of "within" time interval average distance between cases to "between" time interval average distance indicated that "clustering" in the series was weak. Using another approach the clustering appeared to be the

result of a slight excess of pairs within relatively large time or large space distances. The data indicated that a single analysis of a case series with a long time span can be subject to artifactual space-time clustering in the population at risk.

Statistical analysis of epidemiological survey data has provided fairly conclusive evidence that exposure to cats--and particularly sick cats--plays an important role in some cases of leukemia in children.[179] Among 300 children with leukemia and 831 children in a random sample from the same geographical area, the only statistically significant difference (5% level) between cases and controls occurred with exposure to cats. In an effort to clarify these "borderline" results, a mathematical model was developed which took two additional factors into account--a report of illness or death in the cats and a report of any childhood virus. For children exposed to ill or dead cats, the relative risk more than doubled. However, only a relatively small proportion of all cases of childhood leukemia would seem to be accounted for by cat exposures.

A problem of growing concern in all fields of research is that of handling an enormous and continually expanding volume of information. ACORN (Automatic Coder of Report Narrative)[180] demonstrates the feasibility of a linguistically-based automated storage and retrieval system which allows the user to communicate with the system in his own natural language. ACORN is a demonstrational, rather than operational, system in that it is really a prototype. A truly operational system would need many linguistic refinements and retrieval features which are lacking in the current model.

DIAGNOSIS

The major application of human tumor-specific antigenicity at present is in diagnosis. In a study[181] of 279 persons, carcinoembryonic antigen (CEA) was detected in the sera of 32 of 35 patients with carcinoma of the colon, 13 of 13 patients with carcinoma of the pancreas and 6 of 14 patients with other gastrointestinal cancers, confirming previous work[182] which associated circulating CEA with gastrointestinal tract malignancies. The assay may prove useful in predicting persistence or recurrence of tumor after resection of colonic cancer. Six of eight patients with bronchogenic carcinomas, 24 of 46 patients with severe alcoholic liver disease and 7 of 13 patients with uremia also gave positive results, thus requiring careful interpretation of the assay. Another antigenic system (gamma-feto-protein) related to human cancer has been revealed by the Ouchterlony technique.[183] Eight of 1,030 sera from cancer patients precipitated soluble saline extracts from 82 of 115 malignant solid tumors (breast, colon, ovary, kidney, parotid gland, sarcomas of muscle, bone and nerve) and 12 of 16 benign tumors (breast, thyroid, uterus). The serum of 129 of 129 normal subjects (including RH+

and Rh- pregnant women) and of 111 of 111 patients with non-neoplastic diseases did not contain the antibody. Concentrates of fetal human, calf, pig, dog and cat sera (but not newborn or adult sera of these species) contained the antigen. It was not detected in any of 107 human normal tissue specimens but was present in 2 of 55 specimens (jejunum and ileum) with non-neoplastic disease. Antigen was found also in 17 of 200 sera from patients with solid tumors or leukemias. This serological system is not related to the 2 other fetal antigens known to be associated with human cancer; the CEA and the alpha-1-feto globulin of hepatoma.

Immunoglobulin-forming cells, normal and malignant, lack all known surface differentiation antigens of the thymocyte-lymphocyte-axis: TL, θ, Ly-A, Ly-B and MSLA. Two systems of normal alloantigens are expressed on these cells, H-2 and a new differentiation alloantigen designated PC.1.[184] The gene Pca (plasma cell antigen), which specifies PC.1 alloantigen segregates as a mendelian dominant not closely linked with H-2. Since this system is not represented on thymocytes and lymphocytes, it provides a positive marker for both normal and malignant immunoglobulin-forming cells.

Myeloma is the first metastatic human malignancy in which quantitative measurements of the body's burden of malignant cells have been obtained.[185] Quantitative measurements of the total body IgG synthesis rate in vivo and the average myeloma cell IgG synthesis rate in vitro were used to estimate numbers of tumor cells in 10 patients with IgG myeloma. Estimated total body myeloma cell number ranged from 0.5 to 3.1 x 10^{12} myeloma cells and could be correlated with the degree of skeletal damage observed on roentgenograms. This would suggest that the average doubling time of the myeloma cell was 6 months. If the doubling time is constant, this would imply that the disease began with a single cell undergoing malignant transformation approximately 20 years before diagnosis. Serial measurements of tumor cell number may prove useful in characterizing the growth rate and natural history of multiple myeloma. A hyperviscosity syndrome occasionally accompanies multiple myeloma. A concentration- and temperature-dependent aggregation of γG3 proteins apparently plays a major role in this syndrome and may be the explanation for the rapid catabolism and special complement binding of γG3 proteins.[186] The hyperviscosity syndrome develops when γG3 proteins reach a concentration of approximately 4g/100 ml. The phenomenon of hyperviscosity syndrome in γG1 myeloma requires extreme levels of myeloma protein in the circulation. More than 90% of myeloma patients have decreased serum concentrations for at least one normal immunoglobulin component.[187] Of 42 patients with one or more subnormal immunoglobulins who responded to melphalan therapy, significant and sustained improvement occurred in all low components in 21% and in one component in 29%. None of 29 patients unresponsive to melphalan improved low immunoglobulin levels.

When immunoglobulins were in the normal range, decline to subnormal values was always associated with disease progression.

In the aberrant protein synthesis of myeloma there is often excessive production of electrophoretically homogeneous light polypeptide chains appearing in the urine as Bence Jones proteins. Urinary immunoglobulin excretion in patients with various other forms of cancer is within normal limits in most cases.[188] In the few instances where elevated levels of urinary light chains have been discovered there has been a marked predominance of kappa-type light chains. However, they lack the electrophoretic homogeneity of Bence Jones proteins. In another study[189] monoclonal macroglobulinemia occurred in 3.6% of 333 patients with diffuse lymphoma, a prevalence about 60 times more frequent than that in normal subjects. IgM peaks were more frequent in older patients. IgM peaks were found more frequently in patients demonstrating lymph node masses as their major clinical manifestation. There was a close correlation between lymphoma mass and level of the IgM peak in individual patients. These observations support the value of serial serum immunoelectrophoresis in the assessment of disease activity and in confirming the effect of therapy.

Many studies have demonstrated a relationship between activity of enzymes and the pathologic process of tumor growth. Elevation of leucine aminopeptidase (LAP) values in 24-hour urine specimens was present in all 16 cases of disseminated GI tract neoplasms, 19 of 20 cases of disseminated bronchogenic carcinoma, all 6 cases of disseminated cancer of the prostate, all 9 cases of invasive tumor of the genitourinary tract and 12 of 15 cases of various hematologic malignancies.[190] Serum levels versus urine levels were not as consistently elevated. Seventeen patients with localized or surgically cured malignancies had normal LAP values. These results support the clinical value of determination of LAP in estimating the extent of tumor and success of therapy.

A comparison of a group of patients with chronic granulocytic leukemia demonstrating muramidasuria with a similar group not demonstrating the enzyme revealed that all patients with muramidasuria were Philadelphia-chromosome-negative and, conversely, that muramidasuria was not demonstrated in Philadelphia-chromosome-positive patients.[191] Serum muramidase levels were higher in the negative group, and the patients were older, predominantly males, had lower platelet counts, and poorer responsiveness to busulfan than the positive group. In another study,[192] elevated serum muramidase levels were found in 45 patients with polycythemia vera (PV). A high degree of correlation was found between muramidase levels and a high leukocyte count, granulocyte count, serum uric acid, serum vitamin B_{12} content and unsaturated vitamin B_{12} binding capacity. No correlation was found between muramidase levels and hematocrit, monocyte count and leukocyte alkaline phosphatase activity. This appears to support the postu-

late that, in this disease, granulocytes are the prime source of serum muramidase and the elevated levels of muramidase observed are nonspecific and related to the involvement of the granulocyte in the proliferative process responsible for PV.

Urinary 5-amino-4-imidazolecarboxamide (AIC) determination in children with acute leukemia is a reliable indicator of malignant cell activity.[193] AIC levels correlated with marrow blast cell content, less with peripheral blood blasts, and inversely with platelet counts. The data suggest AIC may be useful in differentiating between leukopenia of relapse and that of drug toxicity, and that it may predict leukemic relapse even before diagnostic morphologic marrow changes occur.

Studies of catecholamine metabolism in a patient with neuroblastoma suggest that 3-methoxy-4-hydroxyphenylglycol (MHPG) is a major metabolite of the tumor and that about 25% of this metabolite is further metabolized to 3-methoxy-4-hydroxy-mandelic acid which is rapidly excreted into the urine.[194] A large proportion of the remaining MHPG is excreted as the glucuronide and sulfate conjugates. An assay of urinary MHPG, after hydrolysis of the conjugates may be of value in diagnosis, and in evaluating the effectiveness of therapy in patients with neuroblastoma.

Patients with nonmetastatic renal cell tumors may have high levels of plasma erythropoietin (ESF) while erythrocytosis is found infrequently in these patients.[195] The elevated levels of ESF and erythrocytosis subside upon the removal of the tumor. Patients with renal cysts frequently have higher elevated ESF levels but no erythrocytosis. Elevated ESF levels fall after cyst excision. The levels of ESF activity are higher and are elevated with greater frequency in patients with metastatic renal tumors before and after nephrectomy. Further substantial elevations are noted in these latter patients in response to exogenous androgens. This difference in response to exogenous androgen may distinguish the difference in ESF levels in patients with renal malignancy from those with renal cyst.

Marked disturbances in both the coagulation and fibrinolytic systems were found in a group of 100 unselected patients with disseminated malignancies.[196] In another study, evidence of a thrombotic tendency was present in 32 of 49 patients with acute leukemia.[197] Disseminated intravascular coagulation characterized by defibrination, hemolytic anemia, and thrombocytopenia which occurs occasionally during the course of acute leukemia may also occur as a complication of chemotherapy.[198] Patients with acute leukemia should be routinely evaluated for coagulation abnormalities prior to and during a course of chemotherapy. The results of another study indicate that [^{75}Se] selenomethionine can be utilized in man to estimate fibrinogen and platelet survival simultaneously.[199] The method is relatively uncomplicated and the radiation exposure is low. Further study of this method is in-

dicated for evaluating the physiological role of coagulation mechanisms in the maintenance of normal hemostasis as well as coagulation disorders in various disease states.

The advent of new forms of therapy in the treatment of Hodgkin's disease (HD) requires accurate clinical staging. Laparotomy with splenectomy and liver and para-aortic lymph node biopsies appears valuable for staging of intra-abdominal HD.[200] In unselected, untreated patients with HD without clinical evidence of involvement below the diaphragm, approximately 30% (8 of 27) had histologic evidence of disease, primarily in the spleen and splenic hilar lymph nodes. No instance of liver involvement without concomitant splenic involvement was noted. Left supraclavicular adenopathy appeared to correlate with intra-abdominal disease. Closed needle biopsy of the posterior iliac crest gave additional diagnostic information.[201] Positive biopsy evidence of HD resulted in the restaging from Stage III to Stage IV of 6 out of 49 patients studied. Histologic sections of aspirated bone marrow were far more valuable than smears and were as useful as biopsy, provided that appropriate site was selected for puncture and that sufficient tissue was aspirated.[202] Careful examination of lymph node sections often reveals clues suggesting early involvement of HD.[203] These include: focal obliteration of subcapsular sinuses; foci of inflammatory cells; atypical, malignant-appearing histiocytes and an increase in the deposition of collagen, occasionally in a band-like fashion. Histologic features of HD in sequential biopsy material revealed that histologic subtypes of HD remained constant over long periods of observation in the great majority of cases.[204] When a change in histologic type occurred, it was almost invariably in the direction of greater malignancy rather than the reverse. Observations on the so-called cellular phase of nodular sclerosing HD, in which lacunar cells are conspicuous, but bands of collagen cannot be demonstrated, confirm the suggestion that lacunar cells are probably the most significant feature of this subtype. In five of seven such cases, progression into classical nodular sclerosing HD ultimately did occur, and in two cases no change was observed. The finding of vascular invasion in lymph node biopsy sections from patients with HD regardless of histologic type appears to be a phenomenon highly correlated with extensive disease and short survival.[205]

Elevated serum alkaline phosphatase level is frequently the single objective abnormality in the initial workup of patients with HD who otherwise have no evidence of liver or bone involvement.[206] With advancing stage there was a progressive increase in the percentage of patients with elevated enzyme. Electrophoresis of this enzyme on polyacrylamide gel showed that elevations of hepatic phosphatase were by far the most important cause of serum alkaline phosphatase elevation. In some, but not all cases, the liver isozyme elevation was the result of hepatic lymphoma. Osseous lymphoma was found to be an uncommon cause of alkaline

phosphatase elevation at the onset of HD. The responsiveness of enzyme to successful chemotherapy indicates, in some clinical situations, that it is a satisfactory index of the effectiveness of treatment. Serum iron level, hematocrit, sedimentation rate, and leukocyte alkaline phosphatase level were not reliable indicators of remission or relapse in pediatric patients with HD.[207] That some parameters were still abnormal in remission suggests that there may be a continual interplay between host and basic disease, irrespective of the clinical status.

The Reed-Sternberg (R-S) cell has long been considered diagnostic of HD. The demonstration of cells with the nuclear and cytoplasmic features of R-S cells is necessary but not sufficient for the diagnosis of this disease. The presence of these cells should not be a diagnostic mandate for HD since cells closely resembling or indistinguishable from R-S cells have been found[208] in other benign and malignant lesions: carcinoma of the breast, adenocarcinoma of the lung, melanoma, fibroxanthoma, lymphoma, multiple myeloma, panmyelosis, mycosis fungoides, thymoma, proliferative myositis, infectious mononucleosis and rubeola.

Several studies demonstrate the diagnostic efficacy of radiopharmaceuticals as tumor localizing agents. Evaluation[209] in the first 30 patients with non-ocular melanoma has confirmed the value of the radioiodinated analog of choroquine, ^{125}I-4-(3-dimethylaminopropylamine)-7-iodoquinoline. Studies in dogs suggest the diagnostic potential of 19-iodocholesterol-^{125}I as a localizing agent for photo-scanning the adrenal gland and associated tumors.[210] A technique for scanning choriocarcinoma using radiolabeled antibody has been demonstrated in studies[211] in hamsters bearing transplanted human female choriocarcinomas in their cheek pouches and given ^{125}-I-labeled immunoglobulin G from rabbits immunized with human male chorionic gonadotropic hormone.

Conventional angiographic visualization can be enhanced in some cases, by the administration of epinephrine. In a study[212] of 70 patients with known or suspected pancreatic, hepatic, or biliary neoplasms, the injection of epinephrine into the celiac axis or its branches prior to the injection of contrast material resulted in improved quality of the angiograms in 11 patients. Diagnostic information added with epinephrine angiography included: individual tumor nodules in the liver, metastases in the spleen, extent of pancreatic cancer, recurrent adrenal cancer with liver metastases, intrahepatic bile duct carcinoma and hepatic vein visualization of presence of liver metastases.

Total body thermography can be an aid in the search for early metastatic disease in patients with carcinoma of the breast.[213] In a series of 126 patients there was an 87% true positive rate, 3% false negative rate and 10% false positive rate. Seven false positive thermograms were converted to true positives in

3 to 6 months after the original study. Metastatic liver disease usually carries a grave prognosis. Preliminary evaluation[214] of liver thermography in 68 patients showed: 41 positive, correct; 7 false-positive; 7 negative, correct; 6 false-negative; 7 equivocal. Thermography is inexpensive, consumes little time, causes no patient discomfort and has the added advantage of not requiring exposure to ionizing radiation. Thermography could become an additional aid in diagnosis of metastatic disease as well as a valuable screening technique.

The distribution of DNA along metaphase chromosomes can be visualized in the fluorescence microscope with the aid of fluorescent DNA-binding agents.[215] The fluorescence patterns are then recorded by a reflection photometer. This technique has demonstrated a distinctive fluorescence banding pattern for each chromosome and has made it possible to identify all 24 human chromosome types. Information is accumulating which suggests that some cytogenetic abnormalities in cancer cells are non-random. In a study[216] of chromosome distribution in eight invasive cervical carcinomas there was a reduced proportion of chromosomes in group B in all tumors, and in Group A, D and G in most tumors. Gains were predominantly found in groups C and E, and in two early invasive cases, in group F. Recurring markers included ring chromosomes and an Api chromosome in an early invasive lesion. This latter finding suggests again the possible significance of this particular chromosome at early stages in carcinogenesis. These results suggest that non-random chromosome changes do take place in cervical carcinogenesis and that the nature of these changes may vary with the stage of the disease.

An aneuploid DNA content has been found in a variety of invasive and preinvasive malignant neoplasms analyzed by Feulgen cytophotometry whereas normal tissue and benign neoplasms usually have a diploid to tetraploid DNA content. By analogy with DNA measurements of precancerous lesions of other tissues, the presence of a diploid to tetraploid DNA distribution in endolymphatic stromal myosis suggests that this lesion is not a precursor of endometrial stromal sarcoma but should be placed in the spectrum between benign, noninvasive stromal nodule and the highly malignant stromal sarcoma.[217] In another study,[218] apocrine metaplastic cells in 9 cases of fibrocystic disease of the breast showed that the apocrine-like areas all contained tetraploid cells that significantly increased with increasing histologic grade. In addition, the proportion of tetraploid cells in cases with a papillary proliferation pattern was higher than those with simple cystic formation. The data support the hypothesis that apocrine metaplasia is a proliferative rather than a degenerative process. The data also suggest that some of the "Grade 3 apocrine metaplasias" or ductal papillomatoses with an apocrine-like change may be precursors of carcinoma of the breast.

The mechanical scanner of CYDAC[219] is a practical and sensitive integrating cytophotometer. Systematic differences have been demonstrated in the measured DNA stain content of human leukocytes. It is not known whether these differences reflect DNA inconstancy or whether they can be attributed to cytophotometric error. The operational characteristics of this instrument were analyzed theoretically and measured experimentally and the magnitude of residual errors, particularly as they apply to the measurement of leukocytes, was established. The observed differences in stain content among leukocyte types and among individual cells within types could not be ascribed to either optical or instrumental errors. Thus, the measured differences in the DNA stain content of human leukocytes may result from variations in cellular DNA content, from differences in the stoichiometry of the staining reaction and from modification of the optical properties of the DNA stain complex.

The continuing search for an improved cancer test has focused much attention upon self-obtained specimens for cytologic screening for uterine cancer. A triple blind laboratory evaluation of three tests, vaginal aspiration, cervical scraping, and self-obtained vaginal irrigation, showed that in the presence of squamous carcinoma, the accuracy with the irrigation specimen approached that achieved by both the vaginal aspiration and cervical specimen. The irrigation method detected 74% of the cases of intraepithelial carcinoma while the vaginal aspiration detected 84% and the cervical scraping 96%. The sensitivity of the irrigation sample decreased further with lesser degrees of atypicality. A cancer detection rate of 73% in this series for the self-administered irrigation method as compared to 88% with vaginal aspiration and 91% for cervical scraping methods demonstrates that the irrigation specimen is not a substitute for the other methods.

PHARMACOLOGY

The search for anti-cancer drugs derived from natural sources has been a productive approach which is being continued. For example, chemical investigations begun in the first half of this century showed that toad venoms and squill extracts contain substances having strong effects on heart muscle, potency (90-fold greater than cocaine) as a local anesthetic, and exhibit anti-tumor properties. The active principles upon isolation were found to be bufadienolides, steroids associated with a sugar chain and substituted with an α-pyrone ring. Because of the difficulties involved in forming α-pyrones, none of the bufadienolides could be synthesized, until recently a satisfactory method for cyclization of the vital 5-substituted α-pyrones was developed, a route which was then used for the total synthesis of simple bufadienolides and finally the more complex naturally occurring bufalin. Now, synthetic bufadienolides can be made with minor structural modifications, and tested biologically for their antitumor, cardiac and anesthetic activities.[221]

E. coli is one source of another naturally occurring substance which exhibits antitumor activity. This substance, the enzyme L-asparaginase, was originally thought to afford a unique approach to the chemotherapy of malignant neoplasms by exploiting a metabolic deficiency of tumor cells as contrasted with normal host cells. This, unfortunately, has not held up in the clinic where L-asparaginase treatment is rapidly followed by clinical resistance. In vitro studies have made possible serial determinations of asparagine synthetase activity of bone marrow during L-asparaginase treatment. A decreasing L-asparagine requirement and increasing asparagine synthetase activity were observed, the latter presumably being responsible for the former. This would explain the rapid development of resistance in acute leukemia and also the lack of functional impairment of the normal bone marrow.[222] A more recent report[223] describes the immunosuppressive effect in man. L-asparaginase was found to inhibit the primary immune response, in terms of both humoral antibody production and development of delayed hypersensitivity. In addition, serum of patients receiving L-asparaginase strongly inhibited the in vitro blastogenic responses of the lymphocytes of hematologically normal subjects.

To circumvent the problem of tumor resistance to L-asparaginase, it was hypothesized that if the L-asparagine synthetase activity in resistant tumors could be suppressed by a combination therapy consisting of L-asparaginase and antagonists of glutamine or glutamic acid, a greater retardation of growth of such tumors might be achieved. Accordingly, several animal tumors were selected from those less sensitive to L-asparaginase (the Ehrlich mouse tumor in ascites and solid forms, the mouse melanoma B-16, and the Walker 256 rat carcinosarcoma.) Combination experiments were conducted with L-asparaginase and four antagonists: azaserine; DON (6-diazo-5-oxo-L-norleucine); L-glutamyl-γ-hydrazide; and MSO (DL-methionine-DL-sulfoximine). The results demonstrated the significantly greater antitumor effects of the several combinations as compared with those of each chemical administered alone. It is believed such combinations are of therapeutic interest.[224]

In view of recent evidence suggesting that primary human tumors may be immunogenic, the possibility should be considered that specific tumor antigens may elicit host defensive reactions during the course of human neoplastic diseases. Consequently, a depression of host defenses by anticancer agents might reduce the effectiveness of chemotherapeutic treatments. It is therefore important to establish experimental models capable of assessing the inhibitory action of anticancer drugs upon the host's responses against the very tumors which are the targets of chemotherapy.

A model system for detecting drug impairment of antitumor host defenses was developed taking advantage of the fact that the

complete regression of Sarcoma 180 in vitamin B_6-deficient Swiss mice is due to the immunological response of the host directed against the tumor. By comparing the effects of drug treatments given before or after tumor implantation, it was possible to dissociate the antitumor from the antihost effects of each of 27 drugs tested. This model system uses four response indices: (a) initial tumor growth inhibition; (b) tumor regression during the second week following implantation; (c) mortality in relation to time of death and size of tumors at death; and (d) incidence of complete tumor regression.

Of the 27 drugs tested, 6-mercaptopurine (6-MP) and kethoxal-bisthiosemicarbazone (KTS) inhibited only the growth of the tumor. Both the immunological response of the host against the tumor and the growth of the tumor were inhibited by arabinosyl-cytosine, cyclophosphamide, methotrexate, actinomycin D, uracil mustard, and six other compounds. The host response to the tumor was inhibited by cupric chelate of KTS, 6-aminonicotinamide, 4-deoxypyridoxine, nitrogen mustard, tubercidin, CH_3-G, daunomycin, and six other agents without significant effect on tumor growth.[225]

Several platinum compounds have also been tested against sarcoma-180 as well as against L1210 leukemia. In recent tests, random-bred Swiss mice bearing implants of sarcoma-180 tumor were treated on the 8th day following tumor implant with a single i.v. injection of either cis-platinum (IV) diammonium-tetrachloride or cis-platinum (II) diamminodichloride (4 to 8 mg/kg). They then began to lose weight until about day 12, while tumors remained static in size. After about day 12 or 13, the weight loss was reversed, and the tumors began to become necrotic. When tumor necrosis was complete, the skin formed a flap followed by a scab which over the following week or two healed to a modest scar which in turn disappeared with the regrowth of hair over the area.[226] Although the therapeutic dosage range (4 to 8 mg/kg) is narrow, it was seen that a single injection of 4.0, 6.0 or 8.0 mg/kg of the cis-platinum II diamminodichloride achieved cure rates of 17, 67, and 100%, respectively. A single injection of 10 mg/kg produced about 20% deaths caused by the toxicity of the platinum compound, and higher doses produced an increasingly larger number of deaths. After a period of 6 months, the cured mice appeared to be healthy and without any signs of recurrence of their tumor. The metabolic fate of the injected platinum compounds is currently being studied and the mechanism(s) of action is under investigation. Tests are also in progress to determine whether the cured mice will reject a reimplantation of the tumor.

Occasionally drugs originally used to treat non-malignant diseases will be found to have some antitumor activity. This is particularly true of drugs which interfere with nucleic acid synthesis. Rifamycin, an antibiotic isolated from the fermenta-

tion broths of Streptomyces mediterranei, has been found effective against mycobacteria, trachoma, vaccinia and adenoviruses, and gram-positive and gram-negative bacteria. A derivative, rifampin (formerly designated as rifampicin, also as rifamycin AMP), is clinically useful in certain bacterial infections and can be given orally. This drug appears to act by inhibition of RNA synthesis in intact bacteria, and it specifically inhibits the RNA polymerase reaction in vitro. Recent evidence indicates that rifampin inhibits the RNA polymerase reaction by acting on the enzyme rather than on the DNA template.

A recent study of the effects of rifampin on toxoplasma gondii[227] a protozoan which replicates within vertebrate cells and causes natural infection of many mammals, including man, demonstrated that rifampin, at concentrations of 50µg/ml and greater, significantly inhibited multiplication of toxoplasma in L-cell cultures. Similar concentrations also inhibited growth of L-cells. No effect of rifampin was found on toxoplasma RNA synthesis in toxoplasma preparations free of mammalian cells or on RNA synthesis in L-cells. Mice which had received rifampin in high doses by the oral or intraperitoneal routes were not protected against lethal infection with toxoplasma.

Kinetic measurements of rifampin inhibition of RNA and protein synthesis in both normal and ethylenediamine-tetraacetic acid (EDTA)-treated E. coli indicated that approximately 200-fold higher external concentrations of rifampin were needed to produce a level of inhibition in normal cells comparable to that observed in EDTA-treated cells.[228] The rates of RNA and protein synthesis in both kinds of cells decreased exponentially, after an initial lag phase, at all rifampin concentrations tested. The lag phase was longer and the final exponential slope less for protein synthesis than for RNA synthesis at a given rifampin concentration. Below certain rifampin concentrations, both the lag phase and the subsequent exponential decrease in the rates of RNA and protein synthesis were found to be rifampin concentration-dependent. At greater concentrations only the time of the lag phase was decreased by higher-rifampin concentrations, whereas the slope of the exponential decrease in the rates of RNA and protein synthesis was unaffected. In all cases, the exponential decrease continued to at least 99.8% inhibition of the original rate of synthesis. These results are consistent with the mode of rifampin action determined from other in vitro studies: rifampin prevents initiation of RNA on DNA templates, but not its propagation, by binding the polymerase irreversibly.[228]

Rifampin and several of its derivatives have been tested[228a] for their ability to inhibit the RNA-dependent DNA polymerase associated with RNA tumor viruses. The most effective inhibitor, 2,6-dimethyl-4-N-benzyl demethyl rifampicin, at 100 µg/ml reproducibly blocked 95 to 100% of murine sarcoma virus RNA-dependent ^{3}H-TTP incorporation. The reverse transcriptases of feline leukemia

virus and avian myeloblastosis virus were also inhibited by this drug. Thus, the polymerizing systems of these three RNA tumor viruses possess a common structural feature recognized by the rifampin derivative.

The effect of cationic polyelectrolytes on tumor cells was studied in culture and in mice with transplanted Ehrlich carcinoma and murine leukemia L 5178Y in ascites form. Mice bearing homografts were randomly subdivided into control animals which were untreated, and treated animals which were given s.c. or i.p. injections of different polycations at nontoxic doses. Survival of solid Ehrlich carcinoma-bearing mice was significantly increased by high molecular weight polyethyleneimine (PEI), by polyvinylamine (PVA), and by polypropyleneimine (PPI) at neutral pH, even when administered as late as 5 days after tumor transplant. This was accompanied by a 10 to 40% reduction in takes of the solid tumor. Survival of mice bearing leukemia cells in ascites form was improved only by PPI. Increase in survival also resulted even if the polycation was administered up to 9 days before Ehrlich tumor transplant, with no evidence of weight loss in the host at time of tumor transplant. Thus, cationic polymers may act by nonspecific stimulation of host immune response to transplanted cells as well as by direct electrostatic cytotoxic interaction with tumor cells; they may also inhibit tumor growth directly. Further examination of these possibilities is in progress.[229]

THERAPY

In recent years, just as the electron microscope has made it possible to understand details of cellular microanatomy that were previously obscure and has permitted our rapidly accumulating understanding of biochemistry to be given microanatomical relevance, so too, the advances in the chemistry of nucleic acids have converted this field from one of esoteric biochemical interest into a vast area of knowledge collectively known as "molecular biology". In the absence of any known consistent biochemical abnormality in malignant tissue, the chemotherapeutic attack on the cancer cell must be empirical, and the drugs selected are usually those toxic to rapidly proliferating cells.[230] However, there is an increasing awareness that the successful treatment of malignant disease is predicated on more complete knowledge and application of the numerous and complex factors and anatomical relationships of the malignant cells as they form tumors and disseminate and develop the clinical features collectively recognized as cancer.[231] The empiricism which has characterized chemotherapy and radiotherapy is being replaced by a more rational approach which should facilitate the search for effective combinations not only of drugs, but also of drugs and other therapeutic modalites including surgery.[232]

Combination Therapy

For many types of cancer the value of multiple therapeutic agents is unquestioned. Nevertheless, the differences that are found among the many different types of neoplasms, and the variations seen in the patient which reflect not only host-tumor relationships but also the previous use of therapeutic agents, or the lack thereof, constitute important factors which must be weighed in the selection and scheduling of combination therapy. Many studies illustrate the dimensions of the problems of finding the most effective treatment for the various types of cancer seen clinically.

As the result of carefully planned programs of intensive treatment, the median survival time of children with acute leukemia has continued to lengthen, and the projected five year survival has improved greatly.[233] However, many drugs that are effective in acute leukemia have not been significantly effective in solid tumors of childhood. The dramatic benefit of drug treatment combined with other therapy is illustrated by three case summaries:[231] "Inoperable" Wilms' tumor, neuroblastoma and rhabdomyosarcoma each became operable following judicious combinations of drug treatment, surgery, and irradiation. In many patients with Wilms' tumor, administration of vincristine has been followed by a striking and particularly rapid regression of large tumor masses, sufficient to allow adequate surgical excision. This approach avoids the tissue reactions that follow preoperative radiotherapy and does not compromise the full use of radiotherapy postoperatively. For neuroblastoma, clinical experience indicates that in addition to the surgical and radiotherapeutic attack on the primary tumor, persistent and intensive systemic chemotherapy should be directed toward the distant foci of disease. Cyclophosphamide used alone or in combination with vincristine appears to be an effective drug treatment for neuroblastoma. Some similarities exist between rhabdomyosarcoma and Wilms' tumor. In both, effective surgical and radiotherapeutic modalities can be used and several drugs, namely, vincristine, actinomycin D and cyclophosphamide, can produce significant tumor regression.

Vincristine sulfate (Vcr) combined with radiation therapy has been tested against metastatic Wilms' tumor by the Southwest Cancer Chemotherapy Study Group.[234] Early in the study, 10 of the 22 children received a priming course of Vcr (0.02 mg/kg, i.v. daily for 5 days) followed by 0.05 mg/kg/week for 8 to 12 doses. As the experience with Vcr accrued, the priming course was omitted, and the Vcr dose was increased to 0.075 mg/kg i.v. weekly. Duration of Vcr therapy and the total dosage ranged from 4 to 26 weeks, and from 0.2 mg to 1.4 mg/kg. Radiation therapy was delayed in 20 of the 22 patients from 14 to 41 days in order to assess the effectiveness of Vcr therapy. Radiation dosimetry was determined by the individual therapists at the

participating institutions. For pulmonary disease, 1100 to 2013 rads were given to both lung fields. Those patients with measurable abdominal disease received 1500 to 4000 rads tumor dose to the abdominal site. Complete regression of metastatic disease occurred in 16 (73%) of these 22 patients with Wilms' tumor treated with the combination regimen of Vcr and radiation to the metastases. Ten (45%) of these 22 children are living and well for more than 2 years following treatment of their metastases. The response rate and quality and duration of response to Vcr plus radiation appear similar if not superior to those reported for actinomycin D plus radiation therapy in the treatment of metastatic Wilms' tumor. Further studies are needed to determine optimum drug combinations and schedules to be used in conjunction with radiation therapy in treating metastatic Wilms' tumor.

In describing the work performed over the past 13 years by the Acute Leukemia Group B (ALGB), the term "complementary therapy" is defined to include all forms of chemotherapy applied after remission induction.[239] These include consolidation, intensification, maintenance therapy, and inducer dosing. This last term is defined as the use of drugs ordinarily active for remission induction during the period when the patient is already in remission.

Arabinosyl cytosine was the first drug in the experience of the ALGB to show evidence of maintenance activity in patients with acute myelocytic leukemia (AML). The median duration of remission was 30 weeks, compared with 5 weeks median for a randomly selected group that received no complementary therapy. The fact that those patients who achieve complete remission have longer median survival (14 months) than do those with partial remission (6 months) and that those who enjoy only hematologic improvement survive longer (4 months) than patients who fail to respond (1 month), leads to the belief that when more effective remission induction regimens are found, improvements in complementary therapy regimens will also be developed, to the benefit of all patients with AML.

During the last decade, with the advent of new drugs, techniques, and investigations of the mechanisms of drug action, there has been a progressive increase in survival of children with acute lymphocytic leukemia (ALL). Survivorship at 1, 2, 3, and 4 years is increasing with predictable proportions reaching 5 years without relapse. The first clear delineation of a maintenance activity independent of induction showed[235] a highly significant 3-fold increase in remission duration in those patients who received 6-mercaptopurine versus placebo, for maintenance. In a later study[236], the value of vincristine and prednisone as "inducer doses" for providing considerably longer remissions, comprised the first proof that such inducer treatments exercised an effect on the leukemic cell population even though it was be-

low the threshold of clinical detection. This observation constitutes a central concept in the advance of leukemia chemotherapy which has been made in recent years.[239]

Experiments[237] in mice originally demonstrated the superiority of intermittent methotrexate every fourth day, at doses higher than were possible with continuous therapy. This work was later validated in man[238] and was extended by the ALGB to include vincristine and prednisone as well as methotrexate. The question of whether the schedule or the route of administration of these drugs was the important factor in achieving significantly longer unmaintained remissions was resolved by an ALGB study in which no evidence of effect of route of administration was found.[239]

The development of a bioassay system for estimation of the residual leukemic cellular body burden has provided a means for gross appraisal of the leukemocidal effect of a treatment under study, and has been useful in the design of more effective complementary chemotherapy. One such system,"Regimen D", in which intermittent inducer doses of vincristine and prednisone were interspersed in methotrexate maintenance therapy over an 8-month interval, has achieved subsequent unmaintained remissions exceeding one year in 40% of patients. This provides evidence that human leukemic populations contain cells in G_0 or prolonged G_1 cell cycle phases. It seems possible that after 8 months of therapy selection occurs so that the faster multiplying cell types are eliminated, leaving a slower-growing leukemia.

Complementary therapy is probably not independent of the type of treatment used for induction, although critical experiments which bear on this point have not been reported. However, the interrelationships of drug, dose schedule, first treatment induction, consolidation, intensification, maintenance, inducer drugs, and complicating diseases and their treatment are all considered to be relevant to remission induction, and all these factors are influenced in part by the immunity of the host.[239]

At Children's Memorial Hospital, University of Oklahoma Medical Center, as a member of the Southwestern Chemotherapy Study Group, remission-induction therapy in children with acute leukemia has included since July 1966, 50 induction attempts, 39 of which have been in children with acute undifferentiated leukemia, 7 in acute myelocytic, 3 in acute monocytic, and 1 in acute myelomonocytic leukemia. These involved 25 different patients, 20 with undifferentiated leukemia, 3 with myelocytic and one each with monocytic and myelomonocytic leukemia. Eight different drugs have been used either singly or in different combinations: vincristine and prednisone, methotrexate intravenously, daunomycin and prednisone, daunomycin alone, 6-mercaptopurine and prednisone, vincristine and cyclophosphamide, arabinosyl cytosine and 6-mercaptopurine with 6-methylmercaptopurine riboside.

The parameters for physical findings and symptomatology as outlined in the National Criteria were used to evaluate the quality of response for each patient. Eleven induction attempts in patients with acute monocytic, acute myelocytic, or acute myelomonocytic leukemia resulted in 8 "no responses" and 3 which were unevaluable because of severe complications. From five studies in which five different combinations of drugs were used to induce remission in acute undifferentiated leukemia, the combination of vincristine ($2mg/m^2$/i.v. once weekly) and prednisone (60 mg/m^2/P.O. daily) was used in 29 induction attempts. The drugs were continued in most cases for four weeks, in some patients for five weeks, and in a few, excluding the vincristine, as long as six weeks. Out of this group there were 20 complete remissions at four weeks, and two at six weeks. There were six partial remissions with all patients achieving M-I status. The failure to achieve complete remission was almost entirely on the basis of a low hemoglobin. There was one "no response" in the group, a patient known to be steroid-resistant having relapsed on steroid therapy. Out of this experience the following conclusions were drawn: (1) There is no effective agent for acute leukemias other than the undifferentiated type at the present time. (2) Vincristine and prednisone is an excellent combination for remission induction in acute undifferentiated leukemia, even late in the course of the disease. (3) Prednisone is a critical drug in remission induction and should not be used for maintenance therapy in acute undifferentiated leukemia.[240]

The pediatric Division of the Southwest Cancer Chemotherapy Study Group in 1968 reported the effectiveness of the combined use of vincristine and prednisone for the treatment of children with acute leukemia in relapse who had previously received corticosteroids. More recently this study group reported on further remission induction studies. Sixty-five children who had previously responded completely or partially to concurrent vincristine and prednisone were given a second induction regimen of those two drugs in 4 weekly courses. A complete remission, a complete or partial remission, and a good bone marrow response were obtained in 54%, 75% and 65% of the patients, respectively. Age and sex were unrelated to response. Pronounced peripheral blood deterioration, duration of disease less than 100 days before a second induction attempt, increasing previous antileukemic therapy, and prior cyclophosphamide maintenance therapy were related significantly to a decreasing response rate.[241]

As a sequel to their previous 1970 report, this study group reported on third and fourth remission inductions using vincristine and prednisone for treatment of children 15 years of age or younger with acute leukemia in relapse.[242] Forty-seven children who had received this combination twice previously

were treated for a third remission induction, and of these, 43 completed this study. Eighteen similar children who had achieved three previous remissions on this combination therapy were treated for a fourth remission induction, and of these 16 were evaluable. Prednisone, (60 mg/m^2/P.O daily) was given for 28 days. Vincristine, (2 mg/m^2,i.v. each week) was given for 4 weeks. The criteria for evaluation of bone marrow, hemogram, physical findings and symptoms were those used in previous studies. Following this third course of therapy, 34 of these evaluable patients (79%) achieved M-1 status; 20 patients (46%) achieved complete remission as measured by all criteria, and 18 patients (42%) achieved partial remission, giving a complete plus partial remission rate of 88%. Following this, their fourth course of therapy, 7 of 16 evaluable patients (44%) achieved M-1 status. Only 1 child achieved complete remission, while 8 (50%) achieved partial remission, giving a complete plus partial remission rate of 56% for the fourth induction regimen. Only 3 patients had toxicity sufficient to require a change in dose schedule.

In another cooperative study, daunorubicin was used as the single chemotherapeutic agent for the treatment of 96 children with acute lymphoblastic leukemia resistant to other agents. On a randomized basis, the doses used were 30, 45, or 60 mg/m^2 daily for 5 days, with provision for a second course in those who failed to respond. The marrow remission rates were respectively, 24, 32 and 41% when the three randomized groups were analyzed. When only those who received a full course of drug during the first 5 days of study were examined, the remission rates at both 45 (43%) and 60 mg/m^2 (38%) were superior to that at 30 mg/m^2 (18%). The median time lapse from the first dose to remission was 22 days, with the earliest remission occurring in 11 days. The remissions were short. The median was 66 days (range, 8 to 419 days) in this group of patients. The primary toxicity was hematological with severe leukopenia, thrombocytopenia, and bone marrow aplasia. Cardiac toxicity also occurred. Although the precise mechanism of action of daunorubicin has not been fully elucidated, and the relationship between total dose of daunorubicin administered and cardiac toxicity has not been definitely established, from several previous reports and this clinical study, this cytotoxic antibiotic appears to have definite antileukemic properties. Daunorubicin may find ultimate use as a component in combination therapy for remission induction of acute lymphoblastic leukemia. Further such combination therapy studies are in progress.[2 43]

The proper management of patients with acute leukemia must consider not only the duration or increase of remission time, but also the avoidance of intolerable side effects which in themselves may prevent the patient from enjoying an essentially normal life. A clinical study with 37 patients, 36 of whom had acute lymphoblastic leukemia, and 1 of whom had acute myelo-

blastic leukemia, investigated the relationship between duration of remission and methotrexate dosage. Nineteen patients in a "low dosage" group were given 3 mg/kg i.v. every 2 weeks, and 18 patients in a "higher dosage" group were given larger doses ranging from 3.5 to 6 mg/kg.

From this clinical experience with the "higher dosage" group, it was observed that the dose of the drug must be increased gradually to allow patient's tolerance to the drug to develop in order to avoid drug toxicity. There appeared to be a relationship in these patients between duration of remission and drug dosage. Median duration for the "low dosage" patients was 5.6 months; in the "higher dosage" group it was 11.6 months. The median interval between starting the drug and achieving the final dosage in the high dosage group was 4.4 months. These patients, by having remained in remission long enough to achieve maximal doses, were in a sense, selected, since no difference in toxicity or complications was found between these two groups of patients. This relationship between dose and duration needs further study before the larger dose can be recommended as superior.[244]

The possible beneficial effects of the pyrimidine analogs, 6-azauridine (AzUR) and its 2',3',5'-triacetyl derivative, azaribine (TA-AzUR), in polycythemia vera were suggested by the hematological effects observed in patients with psoriasis, and by earlier clinical pharmacological studies. Five patients with clinically confirmed polycythemia vera were therefore treated with 6-azauridine (180 Mg/kg/day, given in 3 equally divided doses as 2-hour i.v. infusions every 8 hours), or with equivalent amounts of azaribine given orally (270 mg/kg/day, in 3 equal portions every 8 hours). The duration of each course of therapy varied from 3 to 12 weeks and was governed by the clinical and hematological response. One patient received doses of 135 mg/kg/body wt. of TA-AzUR as maintenance therapy for a period of 36 weeks. Allopurinol, (800 mg/day), was used to prevent hyperuricosuria during the first few weeks of therapy.

Amelioration of symptoms, decrease in spenomegaly, and progressive reduction of the hematocrit were uniformly achieved in 3 to 6 weeks without untoward effects. Reticulocytopenia, elevation in serum iron values, and prolongation of plasma iron disappearance time were in accord with the concept that suppression of erythropoiesis is responsible for these beneficial effects. The effectiveness and apparent safety of this inhibitor of pyrimidine biosynthesis, its availability in oral form, its lack of nonhematologic toxicity, and its reversibility recommend continued investigation of its use in the therapy of polycythemia vera. Additional studies in previously untreated patients will be necessary to determine optimum dosage schedules, the value of maintenance therapy, and the effects of these compounds on thrombocytosis.[245]

During the past two decades, the therapeutic spectrum of another antimetabolite, methotrexate (MTX), has widened as its efficacy has been demonstrated in the treatment of acute leukemia of childhood, choriocarcinoma, Burkitt's lymphoma, epidermoid carcinoma of the head and neck region, and also in non-neoplastic disorders as psoriasis, systemic lupus erythematosus and psoriatic arthritis.

Concurrent with the definition of the therapeutic spectrum of MTX, recent studies have sought to improve its therapeutic index. A recent clinical study[246] was designed to use a dose of MTX that would achieve a blood level of 5×10^{6}M of MTX (a level which inhibits DNA synthesis more than 95% in vivo and in vitro); then devise a program of leucovorin "rescue" which would allow this dose of MTX to be given safely for 24 hours with repetition at 4-day intervals; and evaluate the response of patients with advanced carcinoma of the head and neck to this therapy.

Twenty one patients with Stage III or IV epidermoid carcinoma of the head and neck region were treated with 24-hour i.v. infusions of MTX (240 mg/m^2) followed by leucovorin (12 mg. given i.m. every 6 hours for 6 doses, and repeated at 4-day intervals). Thirteen (62%) of these 21 patients responded with a >50% reduction of tumor mass, and 7 of these 13 sustained a >75% reduction. The median of tumor regression was 16 weeks. In general, toxicity was tolerable. The reasons for failure in other patients may have been: poor blood supply, natural biochemical resistance, and tumor growth rate. Those patients whose tumor cells have a significant percentage of their stem cell population in a resting (G_0) state, or whose cells have long generation times would not be expected to respond to this regimen.

The value of antimetabolite therapy is further illustrated by the success which has been obtained with topical 5-fluorouracil (5-FU) ointment (RO-9757) treatment of premalignant keratoses and superficial cutaneous malignant tumors. A recent review, describing the clinical experience with more than 10,000 lesions in over 500 patients, includes the following conclusions:[247] 1) Topical chemotherapy with 5-FU permits selective eradication of skin cancers. Untoward effects on adjacent normal skin or other normal tissues are minimal or absent. Healing occurs with slight or no recognizable scarring, thus reducing or eliminating functional or disfiguring effects. 2)Topical 5-FU is of value for the management of multiple superficial primary skin cancers and precancerous lesions, when surgery or radiation therapy are unsatisfactory because of extensive involvement of the skin by persistently developing tumors. 3) Early primary cutaneous neoplasms may be recognized by their reaction to topical 5-FU before they are otherwise clinically detectable, thus minimizing local tumor spread. 4) Control of neoplasms by topical 5-FU at early stages prevents their progression to more

advanced stages. 5) This local chemotherapy provides evidence that selective eradication of tumors in man by chemical means is feasible.

A prospective randomized study revealed that the combination of radiation plus fluorouracil produced a significantly increased survival in patients with advanced tonsillar and intra-oral cancer, as compared with radiotherapy alone. All patients were staged using the TNM system. Excluded from the series were patients older than 80 years or those whose general condition appeared too poor to tolerate a full course of radiation, those with distant dissemination, and patients who had prior definitive surgery or radiotherapy. There was no significant difference in survival achieved by these two treatment modalities in advanced cancer of the nasopharynx, hypopharynx, or extrinsic larynx, or in primary tumors of unknown origin. Similarly, there was no significant difference in survival resulting from these two methods of treatment in patients with T_1 and T_2 as well as T_4 head and neck cancers, but a highly significant difference in those with T_3 lesions.[248] However, the 66 patients with advanced head and neck cancer treated with radiation alone (6,500 rads over an elapsed time of about 43 days) had a median survival of 14 months, compared with 27 months for the 68 similar patients given 5-FU (5 mg/kg/day on day 4, then 5 mg/kg 3 times weekly throughout the course of radiotherapy) combined with radiation. The percent surviving at 18 months was 40.6% for the group treated with radiation only, compared with 52.7% for combined therapy. Likewise, the differences were quite apparent at 3 years, 18.8% compared with 44.5%, and at 5 years, 15% and 36.1%.

In 1961, the National Surgical Adjuvant Breast Project (NSABP) initiated a randomized prospective clinical trial to assess the worth of postoperative irradiation in the treatment of operable breast cancer. Information was gathered from 1,103 acceptable study patients contributed by 25 institutions. Of these patients, 470 women were recipients of postoperative parasternal, axillary, and supraclavicular irradiation, (a minimum total tissue dose of 3,500 R in not over 3 weeks, or 4,500 R in not over 5 weeks was required), and 633 patients (controls) received either triethylene thiophosphoramide (TSPA), or a placebo.

Disease status and survival rates were determined for all patients 3, 4, or 5 years after operation. No significant difference in these parameters was found at any time between the group receiving radiotherapy and the control groups. However, only 0.6% of the irradiated patients showed regional recurrences as first evidence of disease at 5 years, whereas 8.5% of TSPA and 3.4% of placebo patients showed such recurrence, indicating that irradiation effectively decreased the incidence of regional recurrence in areas irradiated. Likewise, local recurrences were decreased in the radiotherapy group (7.8%) as

compared with placebo-treated patients (15.4%), but not when compared with TSPA controls (5.9%). The proportion of patients whose treatment failures were due to distant metastases as first evidence of disease was greater following irradiation than in the controls (40.0% vs 32.3%). Examination of data concerning axillary nodal (positive or negative) or menopausal status failed to show a clear advantage for the radiotherapy group either in terms of disease status or increased survival. Consequently, it was concluded that this study to the present time has failed to demonstrate clearly the advantage of postoperative irradiation as an adjuvant to surgery in the treatment of operable breast cancer when considered in terms of disease-free status and survival of patients. Observations of patients will continue to determine whether or not an advantage from irradiation occurs after a more prolonged follow-up. This study demonstrated that the occurrence of metastases is an indication of systemic disease, the outcome of which depends upon treatment of the systemic manifestations.[249]

A more encouraging picture may be found in the more comprehensive study which follows: a review of hormone therapy in cancer reveals that during the past twenty-five years standard patterns have become recognized for the management of specific neoplasms, particularly carcinoma of the breast in both men and women, carcinoma of the prostate, and endometrial carcinoma.[250] A sequential pattern of treatment is defined which has been found to provide prolonged control of the cancer. It consists of five phases: 1) mastectomy (radical, if indicated), followed by radiation therapy as indicated by the conventional criteria. 2) primary endocrine therapy. This, for the premenopausal woman, is defined as therapeutic castration, which provides a specific, measurable treatment in which the observed effect is an index of the biological nature of the cancer. For the postmenopausal women, estrogenic hormones produce objective improvement in 35 to 40% of patients so treated, and demonstrate a 2:1 superiority over androgenic hormones. 3) The preferred secondary phase of endocrine therapy is an ablative procedure, either hypophysectomy or adrenalectomy, but should be delayed as long as the patient is asymptomatic. In lieu of surgery, massive doses of adrenocorticosteroids may be given. Cortisone (100 mg three times a day), or its equivalent, may produce objective improvement. This ends the efficacy of hormone therapies. Phase 4, in the presence of progressive disease, must utilize the appropriate cytotoxic chemotherapeutic agents. 5) Supportive therapy: The anti-inflammatory effect of corticosteroids may be used as a supportive measure in the care of the cancer patient. Corticosteroids have effected reduction of intracellular edema in the area surrounding brain metastases or primary brain tumors. Dyspnea caused by metastatic tumors in the lung has been improved. Jaundice has been reduced. Furthermore, steroids are effective in the control of hemolysis second-

ary to specific neoplasms. Massive doses of progestogens, (as 500 mg of hydroxy progesterone caproate three times a week), have induced objective improvement in 25% of women with advanced endometrial carcinoma. Those patients who achieve improvement appear to obtain long-term benefits, often exceeding 10 years. For this reason the use of progestogens, at an adequate dosage, should be tried for at least a three-month interval, and up to 6 months before regarding the treatment as a failure.

In advanced prostatic carcinoma, the principal basis of treatment is the use of antiandrogenic measures: orchiectomy or estrogenic hormones have been the basis of treatment for 30 years. It appears that no benefit accrues from "prophylactic" orchiectomy or estrogenic hormone at the time of diagnosis of early primary carcinoma of the prostate. Surgery is the treatment of choice, and hormone therapy should be reserved for the time when the recurrent disease is symptomatic. Bilateral orchiectomy or estrogenic hormone administration are effective means of producing objective improvement in patients with prostatic carcinoma.

The general principles for control of male breast cancer are similar to those for carcinoma of the prostate in that antiandrogen therapies are employed. Orchiectomy or estrogenic hormone produces objective improvement. Whichever approach is used initially, the other form of therapy is utilized sequentially. Androgenic hormones are never used in the treatment of advanced carcinoma of the breast in men.

Significant numbers of cures of spontaneous mammary adenocarcinoma in CD_8F_1 ♀ (BALB/c ♀ × DBA/8♂) mice have been effected[251] by concomitant administration of three therapeutic modalities: surgery (enucleation) + combination chemotherapy + immunotherapy. The results were obtained following lifetime observation of the animals. Immunotherapy consisted of zymosan (Z), a nonspecific stimulant of the reticuloendothelial system. When Z treatment was begun 3 days prior to enucleative surgery of the spontaneous tumor, it enhanced the therapeutic effect of combination chemotherapy consisting of four simultaneously injected anticancer agents: streptonigrin (S), thioguanine (T), Endoxan (E) or cyclophosphamide, and mitomycin C (M). The first injection of this quadruple combination (STEM) was given on the day after surgery; thereafter, three additional simultaneous injections of the STEM + Z were given at 14-day intervals.

The combination of surgery + STEM + Z effected a 54% local cure rate (i.e. no tumor recurrence) as well as a 24% absolute cure rate (i.e. no tumor recurrence as well as no appearance of a new tumor). Surgery alone, and surgery + STEM produced local cure rates of 15% and 39%, and absolute cure rates of 3% and 11%, respectively. This combination of 2, 3 and 4 chemotherapeutic

agents following surgery produced increased local tumor cure without the toxicity which has been encountered with other regimens. It is generally agreed that the spontaneous tumor experimental system should provide a much more accurate guideline for treatment of human neoplasms than does the well controlled transplantable tumor. It appears reasonable that the rationale should be translatable to more successful therapy of human cancer.

AB-132, [Bis(2,2-dimethyl-1-aziridinyl)phosphinyl]-carbamic acid ethyl ether, is one of a series of drugs, "dual antagonists", in which alkylating groups have been combined with carbamates in an attempt to achieve higher intracellular levels. A previous pilot study led to a clinical trial to determine the effects of AB-132 combined with radiotherapy on survival of patients with either clinically inoperable or demonstrably unresectable pulmonary lesions, who had not yet exhibited evidence of bloodborne metastases. Eighty patients were included in this study, and were randomized in equal numbers between the test treatment and two control groups. All radiation administered to these patients was from a 2 MeV source, HVL 7.6 mm Pb, SSD 100 cm, through equal parallel opposing anterior and posterior fields. A generous margin surrounding all known tumor was included within the treatment fields in all groups. In the treatment group of 40 patients, each was given a total tumor dose of 3,000 rads delivered in 3 weeks, also AB-132 (7.5 mg/kg, iv.) each day that radiotherapy was given. The 20 patients in the second control group received radiation in the same manner except that total tumor dose was 5,000 rads in 5 weeks.

The results of this study were evaluated solely in terms of survival. The major difference observed between patients treated with the combination of AB-132 and irradiation, as compared with those given radiation only, is that 4 of 39 evaluable patients in the test group survived more than 3 years, by which time all control patients had died of their disease. Of the 4 surviving patients, two have survived more than 5 years without evidence of disease, and with only minimal sequelae of treatment. They each had extensive supraclavicular and cervical node involvement. Although much additional study is needed, there is abundant evidence that chemotherapeutic drugs have modes of action sufficiently different from that of radiation to warrant continued clinical studies in the spectrum of human tumors.[252]

In contrast to the foregoing, no beneficial effect from the use of nitrogen mustard (HN_2) as an adjuvant to surgery could be demonstrated in a series of 1,192 patients entered in a Collaborative Surgical Adjuvant Lung Study. The incidence of postoperative complications was high (33%) in the control series but was significantly higher (about 45%) in the treatment series for both the high dose (0.4 mg NH_2/kg) and the low dose (0.3 mg/kg) parts of the study.[253]

Patients with invasive bladder carcinoma present a serious problem that has not yet been resolved satisfactorily. The fact that surgical procedures or irradiation alone each produce survivors has led to studies to find some combination of these modalities that might provide an increase in the incidence of survival. One cooperative study[254] admitted 325 patients with histologically proven, but apparently potentially curable, invasive bladder carcinomas. These patients were randomly selected to receive, or not receive, preoperative irradiation (at least 4,500 R in 28 to 32 days from a supervoltage source). An open surgical procedure, of the surgeon's choice, was done at 4 to 8 weeks following the irradiation, or when irradiation was omitted, was done promptly. Postoperatively, the patients were again randomized to receive either 5-FU or placebo beginning in 14 days. Although operative mortality rates were not influenced by either preoperative irradiation, or by 5-FU, and the only significant increase in non-lethal complications was in wound infection when radiation was used, the 5-FU as used in this protocol showed no advantage in survival and was therefore discontinued.

Another prospective randomized study of 36 patients with advanced bladder cancer was designed to compare the efficacy of radiotherapy alone versus radiotherapy and concomitant 5-FU therapy. An average radiation dose of 6,500 rads given over an average of 47 days was augmented by 5-FU given by the relatively rapid "push-injection" technique, (a "full dose" of 10 mg/kg was given on each of the first 3 days of radiotherapy, followed by "half-doses" of 5 mg/kg, 3 times per week, depending upon clinical indications of toxicity), to a total average dosage of 80 mg/kg. Though no prolonged complications outside the field of irradiation or apparently directly related deaths were encountered, and no significant increase in long-term morbidity was observed over those in patients receiving only radical supervoltage radiotherapy, no significant improvement in over-all patient survival was realized from this combination of 5-FU and radical supervoltage radiotherapy.[255]

It may well be that the best use of 5-FU and supervoltage radiotherapy would be as a pre-planned, midrange, preoperative treatment. This would take into account both the high incidence of local treatment failures with either treatment regimen randomized in this present study as well as the apparent reduction of demonstrable distant metastases in the group receiving combined therapy.

The soft tissue sarcomas vary greatly in their response to ionizing radiation. Two factors that determine clinical curability are the difference in sensitivity between the malignant tissue and the surrounding normal tissue, and the pattern of tumor metastasis. In a clinical study having as its objective the improvement of the cure rate, 8 patients with inoperable malig-

nant soft tissue sarcomas were treated with a combination of methotrexate and radiation either concomitantly or sequentially. Methotrexate was given orally, 2.5 to 7.5 mg daily in divided doses. This dosage was reduced to half if the WBC count fell below 5,000, or the platelet count fell below 100,000/ mm^3. The radiation technic used was designed to include all of the non-resected cancer in the primary radiation beam when possible. A cobalt teletherapy unit was used on ambulatory patients. Hospital in-patients were treated with a 250 KV X-ray source. A daily tumor dose of 150 to 200 rads was given through multiple portals to avoid undue skin reactions. Radiotherapy was continued on a 5 days/week basis and continued for as long as clinical response, or lack of it indicated, for a total radiation dose of up to 6,000 rads over a period of 8 weeks. Experiences with 4 similar patients who received equivalent radiotherapy only, and with 3 who received similar chemotherapy only, were used for comparison.

Survival times, measured from the start of therapy, for those patients given the combined therapy, ranged from 2 to over 48 months, for a median of 19 months. Three of these 8 patients were alive without evidence of disease at intervals of 48, 45, and 29 months, respectively. For those patients who received either radiotherapy or chemotherapy only, survivals ranged from one-half month to over 91 months, but median survival time was only 2 months. Considering the difference in survival in the two groups, a more extensive controlled study seems indicated.[256]

In an attempt to find ways to increase survival of patients with advanced or disseminated colon cancer, repeated courses of 5-FU were used as an adjuvant to surgery between the time of the initial colon resection and second-look re-exploration in a total of 58 patients having transmural involvement, positive regional lymph nodes, clinically palliative resections, and incompletely resectable tumors proven by biopsy. The 5-FU therapy was started three to four weeks postoperatively when the patient was considered metabolically recovered from surgery. Dosage was 12 mg/kg/day for 5 days, and 6 mg/kg on alternative days to the point of mild toxicity. Courses were repeated after an interval of 30 days following the last dose of a preceeding course.

There was no serious drug toxicity and no resultant mortality. Though the small number of cases and short follow-up prevent drawing definite conclusions at present, what was established is that the use of adjuvant chemotherapy and the second-look operation can be done with acceptable mortality and morbidity, and that this approach has merit in converting some patients to a cancer-free state.[257] This investigation is presently being evaluated on a prospective randomized basis by the Central Clinical Drug Evaluation Program.

Heretofore, the radiotherapeutic management of patients with Hodgkin's disease involving pelvic nodes resulted in the sterilization of young female patients through incidental irradiation of the ovaries. To preserve ovarian function in these patients, an operative procedure termed "oophoropexy" has been developed in which, at the same time of a diagnostic laparotomy, both ovaries and their attached vascular pedicles are moved to the midline of the pelvis. Each ovary is then sutured to the serosal surface of either the anterior or posterior wall of the uterus, and metallic clips are placed on the medial portions of the vascular pedicles as markers for future radiological identification. This entire process adds 15 minutes to the total operative time. Following a postoperative recovery period, patients are irradiated with the usual external megavoltage radiotherapeutic techniques, except that of the further shaping of the pelvic field by placing a 10-cm thick lead block between the collimator and the central region of the true pelvis in order to shield the ovaries. Dosimetric measurements indicate that the dose immediately beneath the middle of the lead block is about 9% of the incident dose. Primary transmission accounts for only 0.5% of this; the remainder is due to scattered irradiation which depends primarily upon the area shielded relative to the total field size. In 13 (59%) of 22 patients treated in this manner ovarian function has been preserved, and one of these 13 patients has since conceived and has given birth to a grossly normal baby girl.[258]

Radiation

The historical development of radiation therapy has, of necessity, been based extensively on empiricism. However, in the first three decades of this century radiobiologists have worked closely with radiotherapists to describe and understand the many phenomena produced in biological systems by ionizing radiation. The distinctly different effects of a single dose of radiation versus fractionated doses were demonstrated in classical experiments[259,260] which, though crude and at best only semiquantitative, are the only radiobiological experiments to date that have had a lasting and universal impact on the practive of radiotherapy.

During the era from about 1930 to about 1955, radiological physics and clinical oncology are to be credited with the development of reproducible physical units of radiation dose, of greatly improved radiotherapy apparatus, initially in the kilovoltage and later the megavoltage energy range; of modern techniques of tissue dosimetry, beam alignment, treatment-planning, and of a greatly increasing body of knowledge of the natural history of various neoplasms, their proclivity for and routes of metastasis, and their relative responsiveness to radiation.

In 1957 the possible relevance of the "oxygen effect"[261] to the therapy of human neoplasms containing significant subpopulations of hypoxic cells was pointed out. The radiation literature in the intervening years indicates that the quantitative analysis of tumor and normal tissue radiation responses in vivo is a far more complex problem than had been appreciated; also such processes as recovery, DNA strand breakage and repair, progress in the cell cycle, repopulation, and reoxygenation all play a part in determining response during fractionated irradiation. Although the idea of inducing partial tumor cell synchrony with chemical inhibitors of mitosis, or by utilizing the mitotic delay produced by radiation itself, to achieve cyclic, resonant enhancement of radiation response remains attractive, neither the population kinetic data nor the techniques for sensing parasynchronous fluctuations in the cell cycle in vivo that are essential for its rational exploitation, are at hand. In recent years, through advances in mammalian cell radiobiology, and elucidation of the quantitative effects of cancer chemotherapeutic agents with respect to dose versus survival, relationship of lethal effect to cell cycle, a more rational, less empirical cancer therapy is emerging in which effective combinations of chemotherapy and radiotherapy are now being developed.[232]

The protective effect of hypoxia against the destruction of cell reproductive capacity by irradiation with charged particles of known ionizing density (LET) has been studied systematically in only one line of mammalian cells. To investigate the protective effect of hypoxia further, P-388 murine leukemia cells were irradiated in vitro under aerobic and hypoxic conditions with monoenergetic track-segments of beams of accelerated deuterons or alpha particles at ionization densities (LET) of 6 to 86KeV/μm of unit density absorber, and the survival of their reproductive capacity was assayed in vitro. No significant protection was conferred by hypoxia at the highest LET studied, in contrast to the only similar published studies using human-derived T-1 cells in which significant hypoxic protection was observed up to LETs of 140 to 165 KeV/μm.

A similar difference exists between the responses of murine P-388 leukemia cells and human T-1 cells to irradiation with fast neutrons of different energies. The P-388 cells show a response to fission-spectrum fast neutrons which is essentially independent of the presence or absence of oxygen, and the T-1 cells exhibit an oxygen enhancement ratio (OER) of about 1.5 when irradiated with neutrons of a similar spectrum of energies. While it is becoming increasingly clear that the response to charged particles of known LET is a grossly inadequate predictor for the response of mammalian cells to the complex variety of ionizing events produced by fast neutrons, it is perhaps relevant that about 30% of the total absorbed dose from fission spectrum fast neutrons is due to events of 80 to 150 KeV/μm size, events which the present data would indicate to have an OER around 1.0 for P-388 cells but which have an OER up to 1.7 for T-1 cells.[262]

Miscellaneous

Several recent investigators have observed a possible relationship between growth of a neoplasm and zinc metabolism. Since zinc is required for DNA and protein synthesis, decreased plasma zinc levels in the human host may reflect high zinc requirements of a tumor. To investigate this further, the effect of dietary zinc deficiency on tumor growth was studied in rats using the Walker 256 carcinosarcoma transplantable tumor. The tumor growth data showed that those rats receiving the zinc-deficient diet showed a marked reduction of tumor growth which was accompanied by a striking increase in survival. Adverse effects, as coarseness and loss of hair, dermatitis and scaling of skin, and erratic appetite were observed and their severity was linearly related to the degree of zinc deficiency; antitumor effects can be separated from adverse effects.[263]

These results of inhibition of tumor growth by dietary zinc deficiency have important implications: First, they suggest a unique approach for inhibition of neoplastic growth. Second, by pointing out the importance of one essential metal in tumor growth, they indicate the importance of investigating the role of zinc and other essential metallic elements in malignance cell proliferation.[264]

The control of pain is a part of the total treatment of the patient with cancer. The first step in controlling cancer pain is to develop an understanding of the patient's emotional makeup and to deal with his fears, anxieties and possible misconceptions. The natural history of many types of cancer follows an orderly progression of the disease from one local site to multiple distant sites. Treatment for pain and discomfort should also follow an orderly progression, concentrating initially on the local lesions causing pain and progressing to control of the regional and rarely, generalized pain. Cancer, its treatment, and sequelae may all give rise to discomfort or pain. Hence it is important to establish the specific source of pain. Pain from localized disease can best be treated by surgery, neurosurgery, radiotherapy or, occasionally, chemotherapy. However, most of the analgesic and tranquilizing agents may have undesirable or dangerous side effects such as agranulocytosis, jaundice, obstipation, depressed respiration, and basal ganglion dysfunction. In some instances effects of treatment may give erroneous impressions of the course of disease. For example, jaundice resulting from use of the methylated phenothiazines may be misinterpreted as evidence of liver metastasis and the poor prognosis associated with spread to this organ may deter the physician from undertaking a potentially beneficial course of antitumor therapy. If radiotherapy is not helpful in pain from regional disease, regional nerve block should be considered. Patients in whom neurosurgical procedures will relieve pain should not be allowed to suffer while the physician temporizes.[265]

Prognosis

From the clinical records of the first 1,000 children with malignant neoplasia seen at the University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, there were 78 patients with rhabdomyosarcoma. The prognosis of childhood rhabdomyosarcoma was determined in two ways: First, survival curves were derived; second, in patients with localized disease, the interval from diagnosis to time of local recurrence or appearance of metastases was calculated. The life-table method was used for the analysis of survival and recurrence, and the chi-square test was used for determining associations among age, sex, extent of disease, primary site, and histologic type of tumor.

Analysis of the survival data from these 78 children with rhabdomyosarcoma indicate that age, histology, primary site, extent of disease, and treatment each had a significant influence on prognosis. The best survival occurred in patients with sarcoma botryoides; the poorest with alveolar rhabdomyosarcoma, and the intermediate with embryonal rhabdomyosarcoma. Tumors of the orbit had the best prognosis, and tumors of other structures of the head and neck had the worst prognosis. Children under 7 years of age had better survival curves than those over 7 years. The median duration of survival for the entire group was 19.2 months (5-year survival rate was 35%). The median duration of survival among 54 children with localized disease was 53.3 months; none of the 24 children with extensive or metastatic disease at diagnosis survived as long as 30 months.[266]

In assessing the prognosis and insurability of thyroid cancer patients, numerous factors merit consideration; some of them are peculiarly applicable to tumors that arise primarily in the thyroid gland.[267] A total of 1,192 patients who were treated between 1930 and 1960 were the subjects of prolonged follow-up studies that delineated the pertinent prognostic features. The morphologic tumor type is the single most prognostic feature. End-result studies indicate that well differentiated papillary type tumors have an excellent prognosis providing that the tumors are small and the patient is less than 40 years of age at the time of diagnosis and treatment. When the 5-year survival rates for male and female patients are studied by direct method calculations, the rate for men (83%) was somewhat lower than that for women (88%). Further analysis reveals at least two factors account for this difference: the greater prevalence of large tumors, and the proportion of older patients among the men. About 80% of the women had tumors under 5 cm in diameter, while less than 60% of the men had tumors of comparable size. Likewise, 50% of the women were under 40 years of age at the time of diagnosis, while only 39% of the men were in this age group. These two variables are of prognostic importance and

may readily explain the survival differences between men and women. The difference in survival rates between patients with small tumors and those with large ones was outstanding at each interval (5, 10, 15, and 20 yrs) of recorded follow-up. Similarly, when the age of the patient at time of diagnosis is taken into account, the under-40 age group has a much higher survival during all follow-up periods in which they were observed, 89 to 98% vs 66 to 78%, for the over-40 age group.

Also confirmed is the fact that survival from thyroid cancer is not synonymous with cure, for some patients live for years or even decades with their cancers, despite regional lymph node metastases or even distant spread. In others, the disease follows a rapidly lethal course. Thus, precise data concerning the probable duration of the disease in a given patient are exceedingly difficult to determine; however, these factors (morphologic tumor type, tumor size and patient's age (under or over 40) at time of diagnosis), should have considerable bearing on the insurability of patients in this category.[267]

REFERENCES

1. Miller, J. A. Cancer Res. 30:559-576, 1970. CA-07175 CG TI-CA-5002 CG
2. Gutmann, H. R., D. S. Leaf, Y. Yost, R. E. Rydell and C. C. Chen Cancer Res. 30:1485-1498, 1970. CA-02571 CG
3. Lotlikar, P. D. and L. Luha Proc. Am. Assoc. Cancer Res. 11:50, 1970. CA-10604 CG K4-CA-42,362
4. Scribner, J. D., J. A. Miller and E. C. Miller Cancer Res. 30:1570-1579, 1970. CA-07175 CG TI-CA-5002 CG
5. DeBaun, J. R., J. Y. R. Smith, E. C. Miller and J. A. Miller Science 167:184-186, 1970. CA-07175 CG TI-CA-5002 CG
6. DeBaun, J. R., E. C. Miller and J. A. Miller Cancer Res. 30:577-595, 1970. CA-07175 CG TI-CA-5002 CG
7. Irving, C. C., R. A. Veazey and L. T. Russell Chem.-Biol. Interactions 1:19-26, 1969/70. CA-05490 CG
8. Lotlikar, P. D. and M. B. Wasserman Biochem. J. 120:661-665, 1970. CA-10604 CG K4-CA-42,362
9. Stohrer, G. and G. B. Brown Science 167:1622-1624, 1970. CA-08748 SP
10. Irving, C. C. and L. T. Russell Biochemistry 9:2471-2476, 1970. CA-05490 CG
11. Grunberger, D., J. H. Nelson, C. R. Cantor and I. B. Weinstein Proc. N.A.S. 66:488-494, 1970. CA-02332 CG
12. Fink, L. M., S. Nishimura and I. B. Weinstein Biochemistry 9:496-502, 1970. CA-02332 CG TI-CA-5001 CB
13. Jackson, C. D. and C. C. Irving Chem.-Biol. Interactions 2:261-265, 1970. CA-05490 CG
14. Epstein, S. M., E. L. Benedetti, H. Shinozuka, B. Bartus and E. Farber Chem.-Biol. Interactions 1:113-124, 1969/70. CA-06074 CG CA-10667 CG K3-CA-15,245
15. Hennings, H. and R. K. Boutwell, Cancer Res. 30:312-320, 1970. CA-07175 CG TI-CA-5002 CG
16. Chan, P. C., A. Goldman and E. L. Wynder Science 168:130-132, 1970. CA-08748 SP
17. Jacobs, S. J. and H. S. Rosenkranz Cancer Res. 30:1084-1094, 1970. CA-08374 CB
18. Craig, A. M. and I. Isenberg Proc. N.A.S. 67:1337-1344, 1970. CA-10679 CG
19. Craig, M. and I. Isenberg Biopolymers 9:689-696, 1970. CA-10679 CG

20. Kubinski, H. and C. B. Kasper — CA-08959 CG
Science 171:201-203, 1971. — CA-07175 CG

21. Mizuno, N. S., C. E. Stoops and A. A. Sinha — CA-10298 CG
Nature 229:22,1971.

22. Svoboda, D., J. Reddy and C. Harris — CA-05680 CG
Cancer Res. 30:2271-2279, 1970. — CA-08055 CG

23. Goldblatt, P. J. and R. J. Sullivan — CA-10686 CG
Cancer Res. 30:1349-1356, 1970.

24. Heidelberger, C. — CA-07175 CG
Cancer Res. 30:1549-1569, 1970. — TI-CA-5002 CG

25. Tasseron, J., H. Diringer, N. Frohwirth, S. S. Mirvish and C. Heidelberger — CA-07175 CG
Biochemistry 9:1636-1644, 1970.

26. Sorof, S., E. M. Young, R. A. McBride and C. B. Coffey — CA-05945 CG; CA-06927 SP
Cancer Res. 30:2029-2034, 1970.

27. Litwack, G. and K. S. Morey — CA-10439 CG
Biochem. Biophys. Res. Commun. 38:1141-1148, 1970.

28. Bucovaz, E. T., J. C. Morrison, H. L. James, C. F. Dais and J. L. Wood — CA-01228 CG
Cancer Res. 30:155-161, 1970.

29. Maher, V. M., J. A. Miller, E. C. Miller and W. C. Summers — CA-06519 RAD; CA-07175 CG
Cancer Res. 30:1473-1480, 1970.

30. Bralow, S. P., M. Gruenstein, D. R. Meranze, A. Bonakdarpour and M. B. Shimkin — CA-10439 CG
Cancer Res. 30:1215-1222, 1970.

31. Sugimura, T. and S. Fujimura — NO NCI Support
Nature 216943-944, 1967.

32. Maher, V. M. and W. C. Summers — CA-06519 RAD
Nature 225:68-70, 1970.

33. Fahmy, O. G. and M. J. Fahmy — CA-03188 CG
Chem.-Biol. Interactions 1:257-720, 1969/70.

34. Freese, E. and E. B. Freese — NO NCI Support
Biochemistry 4:2419-2433, 1965.

35. Fahmy, O. G. and M. J. Fahmy, — CA-03188 CG
Int. J. Cancer 6:250-260, 1970.

36. Fahmy, O. G. and M. J. Fahmy — CA-03188 CG
Mutation Res. 9:239-243, 1970.

37. Fahmy, O. G. and M. J. Fahmy — CA-03188 CG
Cancer Res. 30:195-205, 1970.

38. Corbett, T. H., C. Heidelberger and W. F. Dove — CA-07175 CG; TI-CA-5002 CG
Mol. Pharmacol. 6:667-679, 1970.

39. Hatanaka, M. and H. Hanafusa — CA-08747 CB
Virology 41:647-652, 1970.

40. Benjamin, T. L. and M. M. Burger — CA-11473 CB
Proc. N.A.S. 67:929-934, 1970 — CA-10151 CB; K4-CA-16,765

41. Eckhart, W., R. Dulbecco and M. M. Burger — CA-10151 CB, CA-07592 CB, K4-CA-16,765
Proc. N.A.S.68:283-286, 1971.

42. Fried, M. — NO NCI Support
Proc. N.A.S. 53:486-491, 1965.

43. Vogt, M. — CA-07592 CB
J. Mol. Biol. 47:307-316,1970.

44. Cuzin, F., M. Vogt, M. Dieckmann and P. Berg — CA-07592 CB
J. Mol. Biol. 47:317-333, 1970.

45. Levine, A. J. and A. K. Teresky — CA-11049 CB
J. Virol. 5:451-457, 1970.

46. Ritzi, E. and A. J. Levine — CA-11049 CB
J. Virol. 5:686-692, 1970.

47. Maio, J. J.
Personal Communication cited in ref. 46.

48. Smith, B. J. — CA-07592 CB
J. Mol. Biol. 47:101-106, 1970.

49. Brackett, B. G., W. Baranska, W. Sawicki and H. Koprowski — CA-04534 CB, CA-10815 CB
Proc N.A.S. 68:353-357, 1971.

50. Temin, H. M. and S. Mizutani — CA-07175 CG, K3-CA-8182
Nature 226:1211-1213, 1970.

51. Baltimore, D. — NO NCI Support
Nature 226:1209-1211, 1970.

52. Spiegelman, S., A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson — CA-02332 SP, NCI Contract
Nature 227:563-567, 1970.

53. Balduzzi, P. and H. R. Morgan — CA-05206 CB
J. Virol. 5:470-477, 1970.

54. Hanafusa, H. and T. Hanafusa — CA-08747 CB
Virology 43:313-316, 1971

55. Garapin, A.-C., L. Fanshier, J.-A. Leong, J. Jackson, W. Levinson and J. M. Bishop — CA-10223 CB
J. Virol. 7:227-232, 1971.

56. Duesberg, P. H. and E. Canaani — CA-11426 CB
Virology 42:783-788, 1970.

57. Rokutanda, M., K. Fujinaga, H. Rokutanda, R. K. Ray, M. Green and C. Gurgo — NCI Contract
Nature 227:1026-1028, 1970.

58. Spiegelman, S., J. Schlom, A. Burny, M. Travnicek, M. R. Das, K. Watson and J. Keydar — CA-02332 SP, NCI Contract
Nature 227:1029-1031, 1970.

59. McDonnell, J. P., N. Quintrell, A.-C. Garapin, L. Fanshier, W. E. Levinson and J. M. Bishop — CA-10223 CB
Nature 228:433-435, 1970.

60. Mizutani, S., D. Boettiger and H. M. Temin — CA-07175 CB, K3-CA-8182
Nature 228:424-427, 1970.

61.	Weber, G. H., A. A. Kiessling and G. S Beaudreau J. Virol. 7:214-220, 1971.	CA-06999 CB K3-CA-19,449
62.	Kiessling, A. A., G. H. Weber, A. O. Deeney, E. A. Possehl and G. S. Beaudreau J. Virol. 7:221-226, 1971.	CA-06999 CB K3-CA-19,949
63.	Levinson, W., J. M. Bishop, N. Quintrell and J. Jackson Nature 227:1023-1025, 1970.	CA-10223 CB
64.	Schlom, J., D. H. Harter, A. Burny and S. Spiegelman Proc. N.A.S. 68:182-186, 1971.	CA-02332 SP
65.	Lin, F. H. and H. Thormar J. Virol. 6:702-704, 1970.	NO NCI Support
66.	Stone, L. B., E. Scolnick, K. K. Takemoto and S. A. Aaronson Nature 229:257-258, 1971.	NO NCI Support
67.	Parks, W. P., G. J. Todaro, E. M. Scolnick and S. A. Aaronson Nature 229:258-260, 1971.	NO NCI Support
68.	Scolnick, E. M., S. A. Aaronson, G. J. Todaro and W. P. Parks Nature 229:318-321, 1971.	NO NCI Support
69.	Lee-Huang, S. and L. F. Cavalieri Proc. N.A.S. 50:1116-1122, 1963.	CA-03190 CB
70.	Cavalieri, L. F. and E. Carroll Biochem. Biophys. Res. Commun. 41:1055-1060, 1970.	CA-08748 SP
71.	Penner, P. E., L. H. Cohen and L. A. Loeb Biochem. Biophys. Res. Commun. 42:1228-1234, 1971.	CA-11524 CG CA-06927 SP
72.	Temin, H. M. Persp. Biol. Med. 14:11-26, 1970.	CA-07175 CG K3-CA-8182
73.	Huebner, R. J. and G. J. Todaro Proc. N.A.S. 64:1087-1094, 1969.	NO NCI Support
74.	Fialkow, P. J., E. D. Thomas, J. I. Bryant and P. E. Neiman Lancet I:251-255, 1971.	CA-10895 SP
75.	Slifkin, M., L. P. Merkow, M. Pardo, S. M. Epstein, J. Leighton and E. Farber Science 167:285-287, 1970.	CA-06074 CG CA-10667 CG K3-CA-15,245
76.	Teebor, G. W. and I. Seidman Cancer Res. 30:1095-1101, 1970.	CA-10978 CG
77.	Silagi, S. and S. A. Bruce Proc. N.A.S. 66:72-78, 1970.	CA-10095 CB
78.	Pitot, H. C., C. Peraino, C. Lamar, Jr. and A. L. Kennan Proc. N.A.S. 54:845-851, 1965.	CA-07175 CG
79.	Bresnick, E. and U. B. Thompson J. Biol. Chem. 240:3967-3974, 1965.	CA-06571 CB
80.	Bresnick, E., S. S. Williams and H. Mosse Cancer Res. 27:469-475, 1967.	CA-06571 CB

81. Bresnick, E. and S. S. Burleson — CA-10893 CG
Cancer Res. 30:1060-1063, 1970.

82. Desai, L. S. and G. E. Foley — CA-06516 SP
Biochem. J. 119:165-170, 1970. — K6-CA-22,150

83. Turner, G. and R. L. Hancock — CA-10476 CG
Life Sci. 9:917-922, 1970. — TI-CA-5013 CB

84. Kerr, S. J. — NO NCI Support
Biochemistry 9:690-695, 1970.

85. Kerr, S. J. — CA-11286 PH
Proc. N.A.S. 68:406-410, 1971.

86. Liau, M. C., N. C. Flatt and R. B. Hurlbert — CA-10244 CG
Biochim. Biophys. Acta 224:282-285, 1970.

87. Hancock, R. L. and J. Grunau — CA-10476 CG
J. Nat. Cancer Inst. 45:971-978, 1970.

88. Volkers, S. A. S. and M. W. Taylor — CA-11496 CG
Biochemistry 10:488-497, 1971.

89. Mittelman, A., G. B. Chheda and J. Luch — CA-10835 CB
Proc. 10th Int. Cancer Congr. 1970.

90. Mittelman, A. — CA-10835 CB
Cancer Res. In Press.

91. Fiala, S. and A. E. Fiala — CA-07618 CG
Naturwissenschaften 56:565, 1969.

92. Fiala, S. and M. D. Reuber — CA-07618 CG
Gann 61:275-278, 1970.

93. Fiala, S. and A. E. Fiala — CA-07618 CG
Experientia 26:889-890, 1970.

94. Watanabe, M., V. R. Potter and H. P. Morris — CA-07175 CG
Cancer Res. 30:263-273, 1970. — TI-CA-5002 CG
CA-10729 CB

95. Lotlikar, P. — CA-10604 CG
Biochem. J. 118:513-518, 1970. — K4-CA-42,362

96. Wu, C. and H. P. Morris — CA-10729 CB
Cancer Res. 30:2675-2684, 1970.

97. Siperstein, M. D., A. M. Gyde and H. P. Morris — CA-08501 CG
Proc. N.A.S. 68:315-317, 1971.

98. Siperstein, M. D. — CA-08501 CG
Trans.Am. Clin. Climatol.Assoc. 82:107-118, 1970. — CA-05200 CG

99. Siddiqui, B. and S. Hakomori — CA-10909 IM
Cancer Res. 30:2930-2936, 1970.

100. Hakomori, S. — CA-10909 IM
Proc. N.A.S. 67:1741-1747, 1970.

101. Patinkin, D., M. Schlesinger and F. Doljanski — CA-06582 CG
Cancer Res. 30:489-497, 1970.

102. Weiss, L. and L. F. Sinks — CA-07918 CC
Cancer Res. 30:90-94, 1970.

103. Weiss, L. — CA-07918 CC
Wistar Institute Symposium Monograph 8: 73-77, 1968.

104.	Fox, T. O., J. R. Sheppard and M. M. Burger *Proc. N.A.S.* 68:244-247, 1971.	CA-10151 CB K4-CA-16,765 CA-11595 CG
105.	Inbar, M. and L. Sachs *Proc. N.A.S.* 63:1418-1425, 1969.	NO NCI Support
106.	Burger, M. M. and K. D. Noonan *Nature* 228:512-515, 1970.	CA-10151 CB
107.	Rabinowitz, Z. and L. Sachs *Nature* 220:1203-1206, 1968.	NO NCI Support
108.	Inbar, M., Z. Rabinowitz and L. Sachs *Int. J. Cancer* 4:690-696, 1969.	NO NCI Support
109.	Inbar, M. and L. Sachs *Nature* 223:710-712, 1969.	NO NCI Support
110.	zurHausen, H., H. Schulte-Holthausen, G. Klein, W. Henle, G. Henle, P. Clifford and L. Santesson *Nature* 228:1056-1058, 1970.	CA-04568 CB
111.	Gunven, P., G. Klein, G. Henle, W. Henle and P. Clifford *Nature* 228:1053-1055, 1970.	CA-04568 CB
112.	Pearson, G., F. Dewey, G. Klein, G. Henle and W. Henle *J. Nat. Cancer Inst.* 45:989-995, 1970.	CA-04568 CB
113.	Nadkarni, J. S., J. J. Nadkarni, G. Klein, W. Henle, G. Henle and P. Clifford *Int. J. Cancer* 6:10-17, 1970.	CA-04568 CB
114.	deSchryver, A., G. Klein and G. deThe *Clin. Exp. Immun.* 7:161-171, 1970.	CA-04747 CB
115.	Hirshaut, Y., P. Glade, L. Octavio, B. D. Vieira, E. Ainbender, B. Dvorak and L. E. Siltzbach *New Eng. J. Med.* 283:502-506, 1970.	CA-08748 SP
116.	Johansson, B., G. Klein, W. Henle and G. Henle *Int. J. Cancer* 6:450-462, 1970.	CA-04568 CB
117.	Goldman, J. M. and A. C. Aisenberg *Cancer* 26:327-331, 1970.	CA-02421 PH CA-07179 IM
118.	Freedman, M. H., G. S. Gilchrist and G. D. Hammond *J.A.M.A.* 214:1677-1680, 1970.	CA-02649 CC
119.	Adams, R. A., E. E. Hellerstein, L. Pothier, G. E. Foley, H. Lazarus and A. B. Stuart *Cancer* 27:651-658, 1971.	CA-06516 SP
120.	Stonehill, E. H. and A. Bendich *Nature* 228:370-372, 1970.	CA-08748 SP
121.	Coggin, J. H., K. R. Ambrose and N. G. Anderson *J. Immun.* 105:524-526, 1970.	NCI Contract
122.	Blair, P. B. *Cancer Res.* 30:1199-1202, 1970.	CA-05388 CB

123. Charney, J. and D. H. Moore — CA-08740 CB
Nature 229:627-628, 1971.

124. Stockert, E., L. J. Old and E. A. Boyse — CA-08748 SP
Proc. Amer. Assoc. Cancer Res. 11:76, 1970.

125. Boyse, E. A., E. Stockert, C. A. Iritani and L. J. Old — CA-08748 SP
Proc. N.A.S. 65:933-938, 1970.

126. Aoki, T., E. A. Boyse, L. J. Old, E. deHarven, U. Hammerling and H. A. Wood — CA-08748 SP
Proc. N.A.S. 65:569-576, 1970.

127. Romsdahl, M. M. and I. S. Cox — CA-11023 CG
Arch. Surg. 100:491-497, 1970.

128. Romsdahl, M. M. and I. S. Cox — CA-11023 CG
Proc. Amer. Assoc. Cancer Res. 11:67, 1970.

129. Hellstrom, I., K. E. Hellstrom and T. H. Shepard — CA-10188 IM CA-10189 IM
Int. J. Cancer 6:346-351, 1970.

130. Hellstrom, I., K. E. Hellstrom, A. H. Bill, G. E. Pierce and J. P. S. Yang — CA-10188 IM CA-10189 IM
Int. J. Cancer 6:172-188, 1970.

131. Hellstrom, I., K. E. Hellstrom, R. Storb and E. D. Thomas — CA-10188 IM CA-10189 IM
Proc. N.A.S. 66:65-71, 1970.

132. Hellstrom, I. and K. E. Hellstrom — CA-10188 IM CA-10189 IM
Nature 230:49-50, 1971.

133. Hellstrom, I. and K. E. Hellstrom — CA-10188 IM CA-10189 IM
Int. J. Cancer 5:195-201, 1970.

134. Asanuma, Y., A. L. Goldstein and A. White — CA-07470 IM
Endocrinology 86:600-610, 1970.

135. Zesblatt, M., A. L. Goldstein, F. Lilly and A. White — CA-07470 IM
Proc. N.A.S. 66:1170-1174, 1970.

136. Mogensen, B. and F. Kissmeyer-Nielsen — NO NCI Support
Lancet 1:721, 1968.

137. Rudolph, R. H. and E. D. Thomas — CA-10895 SP
Amer. J. Obstet. Gynec. 108:1126-1129, 1970.

138. Sutnick, A. I., W. T. London, I. Millman, V. E. Coyne and B. S. Blumberg — CA-06551 IM CA-06927 SP CA-08069 CB
Med. Clin. N. Amer. 54:805-817, 1970

139. Sutnick, A. I., W. T. London, B. S. Blumberg, R. A. Yankee, B. J. S. Gerstley and I. Millman — CA-06551 IM CA-06927 SP CA-08069 CB
J. Nat. Cancer Inst. 44:1241-1249, 1970.

140. Papac, R. J. — CA-08341 SP
Cancer 26:279-285, 1970.

141. Brody, J. I. and K. Merlie — CA-07000 IM K3-CA-8371 T1-CA-5159 IM
Brit. J. Haemat. 19:193-201, 1970.

142. Robinson, W. A. and B. L. Pike — CA-11305 CB
New Eng. J. Med. 282:1291-1297, 1970.
143. Folkman, J., E. Merler, C. Abernathy and G. Williams — CA-08185 IM
J. Exp. Med. 133:275-288, 1971.
144. Weichert, R. R., III — TI-CA-5108 IM
Amer. J. Med. 49:232-241, 1970.
145. Anderson, D. E., L. S. Pope and D. Stephens — CA-02890 CB, CA-08381 IM
J. Nat. Cancer Inst. 45:697-707, 1970.
146. Ross, M. H., G. Bras and M. S. Ragbeer — CA-06927 SP
J. Nutr. 100:177-189, 1970.
147. DeWys, W., W. J. Pories, M. C. Richter and W. H. Strain — CA-11083 CC, CA-11198 SP
Proc. Soc. Exp. Biol. Med. 135:17-22, 1970. — TI2-CA-8112
148. Hui, Y. H., K. B. DeOme and G. M. Briggs — CA-05388 CB
Proc. Amer. Assoc. Cancer Res. 11:39, 1970. — TI-CA-5045 CB
149. Wynder, E. L. and P. C. Chan — CA-08748 SP
Cancer 26:1221-1224, 1970.
150. Narayan, K. A. — CA-01932 CG
Experientia 26:840-841, 1970. — K3-CA-31,063
151. Barclay, M., V. P. Skipski, O. Terebus-Kerish, E. M. Green, R. J. Kaufman and C. C. Stock — CA-08748 SP
Cancer Res. 30:2420-2430, 1970.
152. MacMahon, B., T. M. Lin, C. R. Rowe, A. P. Mirra, B. Ravnihar, E. J. Salber, D. Trichopoulos, V. G. Valaoras and S. Yuasa — CA-06373 EP
Bull. Wld. Hlth. Org. 42:185-194, 1970.
153. MacMahon, B., P. Cole, T. M. Lin, C. R. Lowe, A. P. Mirra, B. Ravnihar, E. J. Salber, V. G. Valaoras and S. Yuasa — CA-06373 EP
Bull. Wld. Hlth Org. 43:209-221, 1970
154. Mirra, A. P., P. Cole and B. MacMahon — CA-06373 EP
Cancer Res. 31:77-83, 1971.
155. Newell, G. R., S. R. Cole, O. S. Miettinen and B. MacMahon — CA-06373 EP
J. Nat. Cancer Inst. 45:311-317, 1970.
156. Strum, S. B. and H. Rappaport — CA-05183 IM
Pediatrics 46:748-759, 1970.
157. Kessler, I. I. — CA-05165 EP
Cancer 25:510-522, 1970.
158. Kessler, I. I. — CA-06373 EP
J. Nat. Cancer Inst. 44:673-686, 1970.
159. Graham, S., R. Gibson, A. Lilienfield, L. Schuman and M. Levin — CA-11489 EP
Amer. J. Pub. Hlth. 60:266-274, 1970.
160. Shanmugaratnam, K. and C. Y. Tye — CA-08448 CB
J. Chron. Dis. 23:433-441, 1970.

161. Spratt, J. S., F. R. Watson and J. L. Pratt — CA-09741 EP
Dis. Col. Rect. 13:243-246, 1970. — CA-08023 SP

162. Eisenberg, H. and E. Shambaugh — CA-09808 EP
Sixth Nat. Cancer Conf. Proc.:417-420, 1970.

163. Spratt, J. S. — CA-08023 SP
Missouri Med.:835-837, 1970.

164. Stewart, A. — CA-05392 EP
Lancet I:923-924, 1970.

165. Stewart, A. and G. W. Kneale — CA-05392 EP
Lancet II:4-8, 1970.

166. Stewart, A. and G. W. Kneale — CA-05392 EP
Lancet I: 1185-1187, 1970.

167. Wynder, E. L., K. Mabuchi and E. J. Beattie, Jr. — CA-08748 SP
J.A.M.A. 213:2221-2228, 1970.

168. Cole, P., R. R. Monson, H. Haning and G. H. Friedell — CA-06373 EP
New Eng. J. Med. 284:129-134, 1971.

169. Yoshida, O., R. R. Brown and G. T. Bryan — CA-03274 CG
Cancer 25:773-780, 1970. — CA-10017 CG

170. DeGeorge, F. W. and R. R. Brown — CA-03274 CG
Cancer 26:767-770, 1970. — K3-CA-18,404

171. Davis, H. L., J. E. Leklem, I. Carlson and R. R. Brown — CA-03274 CG
Proc. Amer. Assoc. Cancer Res. 11:19, 1970.

172. Bodey, G. P. — CA-05831 SP
Amer. J. Med. Sci. 260:82-89, 1970. — CA-08859 CC
CA-10042 CC

173. Perera, D. R., K. A. Western, H. D. Johnson, W. W. Johnson, M. G. Schultz and P. V. Akers — CA-07594 CC
CA-08480 SP
J.A.M.A. 214:1074-1078, 1970. — TI-CA-5176 PH

174. Rawls, W. E., H. L. Gardner and H. L. Kaufman — CA-04600 CB
Amer. J. Obstet. Gynec. 107:710-716, 1970.

175. Brown, B. W., H. Davis and H. Goodman — CA-11430 EP
Biometrics 26:701-711, 1970.

176. Blumenson, L. E. — K3-CA-34,932
J. Theor. Biol. 27:273-290, 1970.

177. Chang, P. C. — CA-08832 SP
Biometrics 26:749-766, 1970.

178. Klauber, M. R. and P. Mustacchi — CA-05924 EP
Cancer Res. 30:1969-1973, 1970. — CA-09751 EP

179. Bross, I. D. J. and R. Gibson — CA-11531 EP
J. Med. 1:180-187, 1970.

180. Shapiro, P. A. and D. F. Stermole — CA-11531 EP
Computers and Automation 20:1-6, 1971.

181. Moore, T. L., H. Z. Kupchik, N. Marcon and N. Zamcheck
Amer. J. Dig. Dis. 16:1-7, 1970. CA-02090 IM CA-04486 SP

182. Gold, P. and S. O. Freedman
J. Exp. Med. 121:439-462, 1965. NO NCI Support

183. Edynak, E. M., L. J. Old, M. Vrana and M. Lardis
Proc. Amer. Assoc. Cancer Res. 11:22, 1970. CA-08748 SP

184. Takahashi, T., L. J. Old and E. A. Boyse
J. Exp. Med. 131:1325-1341, 1970. CA-08748 SP

185. Salmon, S. E. and B. A. Smith
J. Clin. Invest. 49:1114-1121, 1970. CA-11067 SP

186. Capra, J. D. and H. G. Kunkel
J. Clin. Invest. 49:610-621, 1970. F3-CA-35,190

187. Alexanian, R. and P. J. Migliore
J. Lab. Clin. Med. 75:225-233, 1970. CA-06939 CG CA-03195 CC

188. Lindstrom, F. D., R. C. Williams and A. Theologides
Scand. J. Haemat. 7:383-388, 1970. CA-08832 SP CA-08101 CB CA-05158 PH

189. Moore, D. F., P. J. Migliore, C. Shullenberger and R. Alexanian
Ann. Int. Med. 72:43-47, 1970. CA-03195 CC CA-06939 CG T12-CA-8000

190. Phillips, R. W. and E. R. Manildi
Cancer 26:1006-1012, 1970. CA-11236 CC

191. Perillie, P. E. and S. C. Finch
New Eng. J. Med. 283:456-458, 1970. CA-08287 IM CA-11106 IM

192. Binder, R. A. and H. S. Gilbert
Blood 36:228-231, 1970. CA-04457 CC CA-10728 CC

193. Lulenski, G., M. Donaldson and D. Newcombe
Pediatrics 45:983-995, 1970. CA-08223 CC

194. LaBrosse, E. H.
J. Clin. Endo. Metab. 30:580-589, 1970. CA-08726 PH

195. Murphy, G. P., G. M. Kenney and E. A. Mirand
Cancer 26:191-194, 1970. CA-07745 CB CA-08847 CB

196. Soong, B. C. F. and S. P. Miller
Cancer 25:867-874, 1970 CA-05588 CC

197. Rosner, F., J. V. Dobbs, N. D. Ritz and S. L. Lee
Acta Haemat. 43:65-72, 1970. CA-05923 CC

198. Leavey, R. A., S. B. Kahn and I. Brodsky
Cancer 26:142-145, 1970. CA-08725 CB

199. Brodsky, I., N. H. Siegel, S. B. Kahn, E. M. Ross and G. Petkov
Brit. J. Haemat. 18:341-355, 1970. CA-08725 CB

200. Glatstein, E., H. W. Trueblood, L. P. Enright, S. A. Rosenberg and H. S. Kaplan
Radiology 97:425-432, 1970. TI-CA-5008 RAD CA-05838 RAD

201. Webb, D. I., G. Abogy and R. T. Silver
Cancer 26:313-317, 1970. CA-07968 CC

202. Liao, K. T. — T12-CA-8033
Cancer 27:618-628, 1971.
203. Strum, S. B. and H. Rappaport — T1-CA-5183 IM
Cancer 25:1314-1319, 1970.
204. Strum, S. B. and H. Rappaport — CA-08970 CC
Arch. Path. 91:127-134, 1971. — T1-CA-5183 IM
205. Strum, S. B., G. B. Hutchison, J. K. Park and H. Rappaport — CA-08970 CC; T1-CA-5183 IM
Cancer 25:1304-1313, 1970.
206. Aisenberg, A. C., M. M. Kaplan, S. V. Rieder and J. M. Goldman — CA-07179 IM
Cancer 26:318-326, 1970.
207. Jaffe, N. and Y. M. M. Bishop — CA-06516 SP
Cancer 26:332-337, 1970.
208. Strum, S. B., J. K. Park and H. Rappaport — T1-CA-5183 IM
Cancer 26:176-190, 1970.
209. Boyd, C. M., L. M. Lieberman, W. H. Beierwaltes and V. M. Varma — T1-CA-5134 RAD
J. Nucl. Med. 11:479-486, 1970.
210. Counsell, R. E., V. V. Ranade, R. J. Blair, W. H. Beierwaltes and P. A. Weinhold — CA-08349 PH; T1-CA-5134 RAD
Steroids 16:317-328, 1970.
211. Quinones, J., G. Mizejewski and W. H. Beierwaltes — T1-CA-5134 RAD
J. Nucl. Med. 12:69-75, 1970.
212. Kaude, J. V. and G. W. Wirtanen — CA-06295 RAD
Amer. J. Roent. 110:818-826, 1970.
213. Farrell, C., J. D. Wallace and C. M. Mansfield — T1-CA-5109 RAD
Amer. J. Roent. 111:148-152, 1970.
214. Mansfield, C. M., C. Farrell and S. O. Asbell — T1-CA-5109 RAD
Radiology 95:696-698, 1970.
215. Caspersson, T., L. Zech, C. Johansson and E. J. Modest — CA-06516 SP; K3-CA-22,151
Chromosoma30:215-227, 1970.
216. Auersperg, N. and T. Wakonig-Vaartaja — CA-08748 SP
Acta Cytol. 14:495-501, 1970.
217. Goldfarb, S., R. M. Richart and T. Okagaki — CA-07060 CB; K3-CA-13,975
Amer. J. Obstet. Gynec. 106:524-529, 1970.
218. Izuo, M., T. Okagaki, R. M. Richart and R. Lattes — CA-07060 CB; K3-CA-13,575
Cancer 27:643-650, 1971.
219. Mayall, B. H. and M. L. Mendelsohn — CA-03896 CB
J. Histochem. Cytochem. 18:383-407, 1970. — K6-CA-18,540
220. Coleman, S. A., I. F. Rube, M. Kashgaria and C. C. Erickson — T1-CA-5004 IM
Acta Cytol. 14:502-506, 1970.

221. Pettit, G. R., L. E. Houghton, J. C. Knight and F. Bruschweiler
J. Organic Chem. 35:2895-2898, 1970. CA-10115 PH

222. Ho, D. H. W., J. P. Whitecar, Jr., J. K. Luce and E. Frei, III
Cancer Res. 30:466-472, 1970. CA-05831 SP

223. Ohno, R. and E. M. Hersh
Cancer Res. 30:1605-1611, 1970. CA-05831 SP

224. Tarnowski, G. S., I. M. Mountain and C. C. Stock
Cancer Res. 30:1118-1122, 1970. CA-08748 SP

225. Mihich, E., I. Bross, R. M. Mihich and C. A. Nichol
Cancer Res. 30:1376-1383, 1970. CA-04130 PH CA-11047 PH

226. Rosenberg, B. and L. Van Camp
Cancer Res. 30:1799-1802, 1970. CA-11349 PH

227. Remington, J. S., T. Yagura and W. S. Robinson
Proc. Soc. Exp. Biol. Med. 135:167-172, 1970. CA-10467 CB

228. Reid, P. and J. Speyer
J. Bact. 104:376-389, 1970. CA-11262 CG

228a. Green, M.
Unpublished Results NCI Contract

229. Moroson, H.
Cancer Res. 31:373-380, 1971. CA-08748 SP

230. Yarbro, J. W.
Geriatrics 25:135-148, 1970. CA-11266 PH

231. Sutow, W. W.
Postgrad. Med. 48:173-177, 1970. CA-02501 CB

232. Kaplan, H. S.
Radiation Res. 43:460-476, 1970. CA-05838 RAD

233. Holland, J. F.,
Pediatrics 45:191-193, 1970. Chairman, Acute Leukemia Group B.

234. Vietti, T. J., M. P. Sullivan, M. E. Haggard, T. M. Holcomb and D. H. Berry
Cancer 25:12-30, 1970. Southwest Cancer Chemotherapy Study Group.

235. Freireich, E. J., E. Gehan, E. Frei, III, L. R. Schroeder, I. J. Wolman, R. Anbari, E. O. Burgert, S. D. Mills, D. Pinkel, O. S. Selawry, J. H. Moon, B. R. Gendel, C. L. Spurr, R. Storrs, F. Haurani, B. Hoogstraten and S. Lee
Blood 21:699-716, 1963. Acute Leukemia Group B.

236. Chevalier, L. & O. Glidewell
Proc. Amer. Assoc. Cancer Res. 8:10, 1967. Acute Leukemia Group B.

237. Goldin, A., J. M. Venditti, S. R. Humphreys and N. Mantel
J. Nat. Cancer Inst. 17:203-212, 1956. NO NCI Support

238. Selawry, O. S. and D. James
Proc. Amer. Assoc. Cancer Res. 6:54, 1965.
Southwest Cooperative Chemotherapy Group.

239. Holland, J. F. and O. Glidewell
Recent Results in Cancer Res. 30:95-108, 1970.
Acute Leukemia Group B.

240. Lane, D. M. CA-11233 CC
J. Oklahoma Med. Assoc. 63:498-501, 1970. CA-11843 CC

241. Lane, D. M., M. E. Haggard, D. Lonsdale, K. Starling and M. P. Sullivan
Cancer Chemotherapy Reports, Pt. 1. 54: 113-118, 1970. Southwest Chemotherapy Study Group, Pediatric Division.

242. Starling, K.
Cancer Chemotherapy Rep. Pt. 1, 54:293-294, 1970. Southwest Chemotherapy Study Group

243. Jones, B., J. F. Holland, A. R. Morrison, S. L. Lee, L. F. Sinks, J. Cuttner, A. Rausen, F. Kung, H. J. Pluss, F. I. Haurani, R. B. Patterson, J. Blom, E. O. Burgert, Jr., J. H. Moon, L. Chevalier, A. Sawitsky, M. M. Albala, R. Jackson Forcier, G. Falkson and O. Glidewell
Cancer Res. 31:84-90, 1970. Acute Leukemia Group B.

244. Nagao, T., C. B. Lampkin and A. M. Mauer CA-04826 IM
J. Ped. 76:134-137, 1970. TI-CA-5196 IM

245. DeConti, R. C. and K. Calabresi TI-CA-5138 PH
Ann. Intern. Med. 73:575-579, 1970.

246. Capizzi, R. L., R. C. DeConti, J. C. Marsh and J. R. Bertino CA-08341 SP K3-CA-8853
Cancer Res.30:1782-1788, 1970. TI-CA-5138 PH

247. Klein, E., H. L. Stoll, E. Miller, H. Milgrom, F. Helm and G. Burgess CA-06695 PH CA-08678 PH
Dermatologica 140. Supp. 1: 21-33, 1970.

248. Ansfield, F. J., G. Ramirez , H. L. Davis, Jr., B. C. Korbitz, H. Vermund and F. F. Gollin CA-06295 RAD CA-06794 RAD
Cancer 25:78-82, 1970.

249. Fisher, B., N. H. Slack, P. J. Cavanaugh, B. Gardner and R. G. Ravdin CA-05848 SURG CA-10377 CC
Ann. Surg. 172:711-732, 1970. CA-10378 CC

250. Kennedy, B. J. CA-08832 SP
Geriatrics 25:106-112, 1970. TI-CA-5158 PH TI2-CA-8101

251. Martin, D. S., P. E. Hayworth and R. A. Fugmann CA-04497 PH
Cancer Res. 30:709-716, 1970.

252. Webster, J. H., J. E. Sokal, C. A. Ross, H. A. Velasco, J. L. Ambrus and L. Stutzman CA-07800 IM
Cancer Bull. 22:59-61, 1970.

253. Slack, N. H. — CA-10331 CC
Cancer 25:987-1002, 1970. Collaborative Surgical Adjuvant Lung Study — CA-10377 CC, CA-10378 CC

254. Prout, G. R., Jr., N. H. Slack and I. D. J. Bross — CA-06732 CC
J. Urol. 104:116-129, 1970.

255. Edland, R. W., J. B. Wear, Jr. and F. J. Ansfield — CA-06295 RAD, CA-06749 SP
Amer. J. Roentgen. 108:124-129, 1970.

256. Brenner, S. M., S. P. Miller and D. Custodio — CA-05588 CC
New York J. Med. 70:855-860, 1970.

257. Mackman, S., A. R. Curreri and F. J. Ansfield — CA-06071 SURG, CA-06749 SP
Arch. Surg. 100:527-534, 1970.

258. Ray, G. R., H. W. Trueblood, L. P. Enright, H. S. Kaplan and T. S. Nelsen — CA-05838 RAD, TI-CA-5008 RAD
Radiology 96:175-180, 1970.

259. Regaud, C. — NO NCI Support
C. R. Soc. Biol. 86:787-790, 1922.

260. Regaud, C. and R. Ferroux — NO NCI Support
C. R. Soc. Biol. 97:431-434, 1927.

261. Gray, L. H. — NO NCI Support
Brit. J. Radiol. 30:403-406, 1957.

262. Berry, R. J. — CA-10059 RAD
Radiation Res. 44:237-247, 1970.

263. DeWys, W., W. J. Pories, M. C. Richter and W. H. Strain — CA-11198 SP
Proc. Soc. Exp. Biol. Med. 135:17-22, 1970. — TI2-CA-8112, CA-11083 CC

264. McQuitty, J. T., Jr., W. D. DeWys, L. Monaco, W. H. Strain, C. G. Rob, J. Adgar and W. J. Pories — CA-11198 SP, TI-CA-5108 IM, TI2-CA-8112
Cancer Res. 30:1387-1390, 1970.

265. Hall, T. C. — CA-11083 CC
Postgraduate Med. 48:223-227, 1970

266. Sutow, W. W., M. P. Sullivan, H. L. Ried, H. G. Taylor and K. M. Griffith — K6-CA-2501
Cancer 25:1384-1390, 1970.

267. Frazell, E. L., D. Schottenfeld and R. V. P. Hutter — CA-08748 SP
CA. 20:270-275, 1970.

Statistical Summary of NCI Extramural Awards

	F. Y. 1970 (Actual)		F. Y. 1971 (Estimate)	
A. Research Grants	Number Projects	Amount $	Number Projects	Amount $
Total Research Grants	1,237	72,032,000	1,537	89,510,000
General Research Support	- -	6,430,000	- -	5,901,000
B. Training				
Postdoctoral Felloswhips	121	871,812	110	796,000
Special Fellowships	59	727,441	71	872,000
Graduate Training Grants	87	5,244,633	88	5,317,000
Clinical Cancer Training Grants	103	5,456,998	103	5,457,000
C. Research Career Program				
Research Career Awards	11	331,062	11	331,000
Research Career Development Awards	68	1,587,755	75	1,799,000
Grand Total	1,686	92,681,701	1,995	109,983,000

SUMMARY STATEMENT
ANNUAL REPORT
July 1, 1970 through June 30, 1971
LABORATORY OF BIOCHEMISTRY

The LABORATORY OF BIOCHEMISTRY currently consists of five sections containing in the aggregate 15 permanent independent investigators and 15 investigators in the categories of Research Associate, Staff Fellow, Post-Doctoral Fellows, and Guest Worker. The Laboratory conducts research on the regulation of biosynthesis in animal cells, on the identification and characterization of biologically important macromolecules, and on biochemical events and control factors involved in neoplastic transformation, tumor regression, and various instances of normal cell differentiation.

Within the PROTEIN CHEMISTRY SECTION, Dr. E. A. Peterson (Head) has established the feasibility of chromatographing proteins and other macromolecules by displacement rather than elution. Mixtures of polyanionic dextrans having a suitable range of affinities for DEAE-cellulose have been shown to be capable of displacing serum proteins (used as a model mixture) to produce chromatographic fractions having unusually high protein concentrations. In addition to the advantages of improved resolution and greater convenience of operation, the technique greatly expedites the successive application of chromatography and electrophoresis on a semi-micro scale. Dr. Peterson is currently studying the reaction conditions required to produce displacers with the desired affinities. This technique promises to have broad application to the separation of biologically interesting macromolecules. For his role in the earlier development of the cellulosic ion exchangers, Dr. Peterson was co-recipient (with Dr. H. A. Sober) of the 1970 Hillebrand Award from the Washington Chapter of the American Chemical Society.

The PROTEIN CHEMISTRY SECTION also continues to support a broad study of the biochemical factors regulating growth and maturation of leukocytes. This program involves the collaborative efforts of Drs. W. H. Evans, S. R. Himmelhoch, and M. G. Mage with the current support of Drs. R. K. Desser, and T. Gessner (Research Associates), C. Grieshaber and F. Huang (Guest Workers), and others. It is intended to provide information relevant to the normal course of leukocyte differentiation as well of derangements of maturation encountered in various types of leukemia.

A major aspect of this study has been the isolation and characterization of the leukocyte granule proteins as biochemical markers of maturation. Previous annual reports of this project have described the resolution of the neutrophilic and eosinophilic myeloperoxidases (CM-1 and CM-2) from guinea pig bone marrow, as well as the isolation of the leukocyte alkaline phosphatase. In the past year, highly purified neutrophil and eosinophil granules have been prepared and their constituent proteins resolved by gel electrophoresis, immunodiffusion and ion-exchange chromatography. A detailed description of the number, properties and peculiarities of the

normal protein constituents of these granules is being compiled, in order to permit comparison with those found in immature and leukemic cells. A new method for isolating eosinophil peroxidase in high yield has been developed, overcoming difficulties imposed by peculiar physical properties of this enzyme. High purified human leukemic (i. e., neutrophil) myeloperoxidase has been shown by electron microscopy and electrophoresis to consist of two similar subunits (M. Januska and Dr. E. Shelton). Two atoms of iron are present per mole of protein. This protein is being subjected to extensive physical and chemical analysis.

On the more biological side, cell culture conditions have been established (by Mrs. S. Wilson) which permit in vitro maturation of guinea pig bone marrow granulocytes, e. g., a 2-3 fold increase in mature granulocytes and a 50% increase in alkaline phosphatase activity over a 96 hour culture period. Studies on the synthesis of the granule enzymes are continuing. A search has been undertaken for factors in serum which might have possible regulatory effects on granulocyte maturation. Factors stimulating DNA and RNA synthesis in bone marrow cells in 24 hour stationary cultures have been detected in sera from normal, hypertransfused, and leukopheresed guinea pigs. Investigations are now underway to determine whether the syntheses of specific granulocyte proteins are affected by these stimulatory factors. A study of glycoprotein synthesis in granulocytes during maturation has been initiated.

Dr. M. G. Mage has altered the emphasis of a project previously concerned with the separation of immunocytes by specific immunochemical methods. In studies aimed at the isolation of immunoglobulin messenger RNA, he has shown that lymph node polyribosomes from immunized rabbits can be specifically precipitated by sheep antibodies to rabbit immunoglobulin. He is now analyzing the specific polysome-associated RNA from isotopically labelled cells preparatory to hybridization studies with lymph node DNA. This work is concerned with the molecular basis of immunocyte differentiation and antibody variability.

Dr. O. W. McBride has continued a study initiated last year with Dr. Ozer aimed at the transfer of intact genetic information in the form of chromosomes to mammalian cells in culture. This procedure, particularly when combined with chromosome fractionation techniques, should have certain advantages over cell:cell or nucleus:cell fusion for genetic mapping and for the study of viral integration and rescue. During the past year, improvements have been achieved in the yields of metaphase chromosomes from Chinese hamster cells and in their size separation. Conditions for maximizing uptake of chromosomes by cells (time, temperature, growth stage, etc) have been examined. Azaguanine-resistant cell lines (Chinese hamster or mouse) deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) are recipients for chromosomes isolated from HGPRTase positive cells, successful transfer being evaluated by ability of recipients to grow in selective media. Apparent transformants have been

observed in chromosome-treated cells at a frequency of about 10^{-6}, somewhat greater than the reversion rate observed in control cultures. However, conclusive proof will depend upon demonstrating that the PRTase in recipient mouse "transformants" is of the donor (hamster) type. Such studies are in progress.

The research of the NUCLEIC ACIDS SECTION, Dr. W. C. Schneider (Head) covers a broad range of interests at the basic level, with the ultimate aim of relating the results to the neoplastic cell. Dr. Schneider has continued studies on DNA associated with the cytoplasmic endoplasmic reticulum (microsomes). Previous work (see earlier annual reports) had indicated that liver microsomal DNA and nuclear DNA were identical except for the rates at which ^{3}H-thymidine was incorporated and the half-life of the incorporated radioactivity. Present studies, involving treatment with phenobarbital, hydroxyurea, or the hepatocarcinogen, 3'-methyl-dimethylaminoazobenzene, have not yet succeeded in establishing whether this DNA is derived from the nucleus and/or whether it can be implicated in the carcinogenesis process. The latter possibility is particularly worthy of study in view of the known role of microsome-associated enzymes in conversion of chemical carcinogens to forms reactive with cellular DNA.

Dr. W. R. Kidwell (Staff Fellow) is studying the replication of nuclear DNA, particularly with regard to regulation of chain initiation. His work this year has shown that the synthesis of satellite DNA in cultured mouse cells occurred both early and late in the S-phase of synchronized cell populations, with little or no synthesis of this DNA species in the second quarter of S. The same pattern was observed in nuclei isolated from S-phase cells, replication in vitro appearing to be a continuation of the normal DNA synthetic process. Factors which preferentially stimulated satellite synthesis were found in cytoplasmic extracts and in highest concentrations in cells whose satellite DNA synthesis was in progress (early and late S). Continued efforts will be directed to elucidating the nature of the cytoplasmic signals which initiate nuclear DNA synthesis in a specific temporal sequence.

Dr. E. P. Anderson, Dr. T. Q. Garvey (Research Associate) and Mr. A. Liacouras are continuing studies on the synthesis of uridine nucleotides because of the key role of these compounds in RNA and DNA formation and also because of the sensitivity of certain tumors to uridine analogues. This year, the reaction mechanism of uridylate kinase, 300-fold purified from Tetrahymena, has been investigated in detail. A "ping-pong" mechanism of sequential binding of reactants has been ruled out; a ternary complex of the enzyme with both substrates is thus an intermediate. At the same time work is proceeding on the isolation of uridine kinase from fluorouridine-sensitive mouse ascites tumor cells, with the aim of exploring the molecular parameters of this enzyme, which is known to be subject to feed-back inhibition. Once the mechanisms of action of these enzymes are understood in both drug-resistant and drug-sensitive tumor cells, a more rational approach to chemotherapy may be possible.

Dr. J. Rotherham has completed one phase of her work on the effect of vitamin B_{12} and folic acid on the utilization of thymidine and other nucleic acid precursors by a neoplastic mouse tissue culture line. In collaborative study with the Tissue Culture Section, Laboratory of Biology, one function of vitamin B_{12} appeared to be that of increasing DNA-thymidine synthesis by raising limiting levels of certain folate co-enzymes required in metabolism of one-carbon "fragments". Comparable studies in nontransformed cells are contemplated.

Dr. Otani has completed a study of four enzymes and their isozymes in normal rat liver and four Morris hepatomas. These enzymes were selected because they occupy key positions in pathways between amino acid and carbohydrate metabolism and could therefore be important in controlling the levels of exogenous amino acids required for protein synthesis. Dr. Otani is also working on a simplified synthesis of β-hydroxy amino acids in high yields. These compounds could be useful as chemotherapeutic agents if they serve as antagonists of the natural amino acids in protein synthesis or other amino-acid-requiring reactions.

In the BIOSYNTHESIS SECTION, Dr. E. L. Kuff (Head) and Mrs. K. Lueders have continued investigation of intracisternal A-particles, virus-like structures that occur frequently in mouse tumors and occasionally in normal mouse tissues. Collaborators have included Dr. N. Wivel, Laboratory of Cell Biology, NCI and Drs. H. Ozer and S. Wilson of this Section. By chemical and immunological criteria it has now been possible to define these particles as a group and to differentiate them from the known oncogenic RNA viruses which they resemble morphologically. The A-particles exhibit a remarkable structural stability and contain a common or group specific antigenic reactivity when examined in a variety of mouse tumors. Their major protein components differ markedly in molecular weight from those of the murine leukemia and mammary tumor viruses. The particles exhibit a very low level of DNA polymerase activity, and it is not yet clear whether this is specifically RNA-dependent. Isolated particle fractions were earlier shown to contain RNA, and the nature of this RNA is now under study.

Dr. E. Brad Thompson, together with Drs. S. R. Levisohn and J. V. Miller, Jr. (Research Associates), D. Aviv (Guest Worker) and a number of other collaborators, is investigating various aspects of the control of specific protein synthesis in mammalian cells, using as an experimental system the induction of tyrosine aminotransferase (TAT) by steroid hormones in hepatoma tissue culture (HTC) cells. Dr. Miller has apparently succeeded in partially purifying by affinity chromatography ribosomes specific for TAT synthesis. This work not only implies that the nascent TAT chains fold sufficiently to bind tightly to their cofactor, but may provide a cell-free system for studying translational control in enzyme synthesis. Several projects in cellular genetics of the HTC cells are in progress in an effort to clarify the regulatory mechanisms for TAT induction. Thus, a clonal

variant, HTC-S, which is consitutive but poorly inducible for TAT has been isolated by Dr. Levisohn. Also, drug-resistance (selective) markers have been developed in HTC and other cell lines for use in cell fusion experiments. It has now been shown that non-inducibility is rapidly dominant to inducibility when HTC cells are fused with TAT-negative cells, suggesting the presence of control molecules. Other experiments are now in progress on the formation of contact-inhibited cell lines through the fusion of non-contact-inhibited parental cells.

Dr. E. Shelton, in collaboration with other investigators, has demonstrated by electron microscopy a tetrameric radial symmetry for the IgM-like macroglobulin of certain fish, clarifying the relationship of these proteins to the IgM's of higher animals (5-fold symmetry) and providing a basis for tracing gene pools during evolution. She is presently engaged in an ultrastructural analysis of complement component C1q.

As her major effort, Dr. Shelton is developing a program for examining the interaction of lymphocytes and macrophages cooperating in the immune response. By using tracer antigens visible at the ultrastructural level, she hopes to follow these interactions during the secondary antibody response in cells growing in Millipore diffusion chambers (she has previously shown that such chambers implanted by laparotomy in mice permit long-term culture of macrophages and lymphocytes).

Dr. H. L. Ozer (Senior Staff Fellow) has been studying three related aspects of cell interaction with oncogenic virus: 1) the synthesis and assembly of SV 40 virus in productively infected cells; 2) the analysis of temperature sensitive mutants of SV 40 (collaboration with Dr. P. Tegtmeyer, Case-Western Reserve Medical School); and 3) the isolation of temperature sensitive mutants in the mouse cell line, BALB-3T3 (with Dr. R. Wittes, Research Associate). For the SV 40 work, Dr. Ozer has developed techniques which permit examination of intracellular virions, related particles (empty virus shells), and soluble capsid protein, during the course of infection. He has been able to elucidate the normal assembly process and also to classify the mutant viruses according to the type of block observed at the non-permissive temperatures. The later observations will become increasingly important as Dr. Tegtmeyer proceeds with transformation studies on these mutants. Work with the 3T3 cells is aimed particularly at the selection of conditional lethal mutants defective in DNA synthesis. Wild type 3T3 cells are susceptible to transformation by DNA virus (SV 40) and RNA viruses (murine leukemia and sarcoma), and also can support productive infection with polyoma. The mutants should thus be valuable in evaluating host functions during transformation by and replication of oncogenic viruses. Experiments to data have permitted development of procedures for mutagenesis of cells, selective killing of temperature resistant (wild-type) cells by radioactive thymidine, and assay of viral and cell DNA synthesis.

Dr. S. H. Wilson (Senior Staff Fellow) joined the Biosynthesis Section

in the Fall of 1970, to work on the mechanism and regulation of RNA synthesis in nucleated cells. In the early months, he was involved in completing development of a new technique for the radioassay of gas-producing reactions, and he has now published this work. In addition, he has collaborated in the biochemical analysis of avian myeloblastosis virus (with Dr. K. Stromberg, Laboratory of Biology, NCI) and mouse intracisternal A-particles (see above). Simultaneously he has been working out various technical aspects of his project which involves the development of a cell-free system for specific synthesis of ribosomal RNA and the evaluation of protein and other factors in regulating this synthesis. It is anticipated that this study will be facilitated by the availability of temperature-sensitive mutants defective in ribosomal RNA synthesis. Yeast (Saccharomyces cerevisiae) is being used as a model system in the initial phases of the study because of the relative simplicity of its genetic complement and the highly advanced level of genetic analysis which is permitted.

The work of the TUMOR PHYSIOPATHOLOGY SECTION, Dr. P. M. Gullino (Head) is concerned with mammary tumors, and in the past year was concentrated on the problem of tumor regression. Two interrelated effects were studied: 1) the definition, in physiopathological terms, of the regression process (Dr. Gullino); and 2) the role of lysosomal enzymes as causative agents of tissue involution (Dr. Cho-Chung, Visiting Fellow, and Dr. Lanzerotti, Research Associate).

Hormone dependent rat mammary tumors (primary 7-12 DMBA-induced and transplantable MTW 9) were used as models. Histologically, the regression process appears to start without an obvious change either in the morphology or in the types of cells of the tumors. The gross chemical composition of tumors regressed to 1/2 original size was roughly the same as that of the growing tumor. DNA-content per nucleus, and cytoplasmic protein:DNA was unchanged. The efficiency of the vascular bed of the regressing tumor was equivalent to that of the growing tumor. The in vivo consumptions of oxygen and glucose per cell DNA were at least as great in regressing as in growing tumors. During the first hours of hormone deprivation, incorporation of ^{3}H-uridine into RNA and ^{14}C-glycine into proteins and lipids increased sharply; in tumors regressed to 1/2 initial size, these rates had fallen to about one-third those seen in the growing state. These and other data suggest that reduction of tumor mass occurs as a coordinated disassembly which starts as soon as the hormone support is withdrawn but is reversed whenever the proper stimulation is resumed. Regression requires consumption of oxygen and the synthesis of RNA and probably of protein and lipids.

In addition, the activities of 6 lysosomal enzymes were all increased in the regressing compared to growing tumors. These increases were not due to macrophage infiltration, and they were blocked by either actinomycin D or puromycin, indicating that they reflected new protein synthesis. Experiments to confirm this hypothesis and to explore the role of the enzymes in

the regression process are in progress.

Plans for future work include analysis of the role of estrophilic proteins in the regression process, and comparisons between regression due to hormonal deprivation and cell death resulting from chemosuppression of some metabolic function.

Research within the CYTOCHEMISTRY SECTION, Dr. D. Burk (Head) has been confined largely to the efforts of Dr. M. W. Woods, working in collaboration with Mr. G. Vlahakis, Laboratory of Biology, NCI, and in consultation with Dr. Burk. Mouse strains such as C3H, C3HfB, C3HA^{vy}, and C3HA^{vy}fB, with a high incidence of "spontaneous" hepatomas, have provided very interesting material for studying metabolic changes attendant on malignant transformation in vivo. Also, transplantable liver tumor lines recently established by Mr. Vlahakis facilitate evaluation of metabolic stability during continued growth. "Glycolytic profiles" of a large number of primary and transplanted hepatomas have been compared with one another, and with those of adjacent non-transformed liver and livers of tumor-free animals. Almost all of the tumors showed significant increases in anaerobic glucolysis compared with non-tumor liver tissue. As a standard procedure, glycolysis was measured at two glucose levels (0.28% and 2.8%); tumors consistently showed a lower fold of stimulation by high glucose, consistent with an increased affinity for glucose compared to normal liver. The response to anti-glucolytic steroids and/or other agents will be compared in future work. The data are consistent with the hypothesis that "glucolytic capacity, measured anaerobically in vitro, is critically related to tumor growth potentials in the intact animal and may be a major point of control."

In other activities within the section, Dr. Burk has apparently confirmed by a biological test that a particular mechanical device dilutes cigarette smoke with air in accordance with the manufacturer's claims (NCI Project No. 330). Some of this work, and extrapolated conclusions, has been published in the form of a signed endorsement in public advertisements of the device. In addition, Dr. Burk reports that "amygdalin, prunasin, mandelonitrile, and cyanide-benzaldehyde, in the order named, produce increasing inhibitions of the Pasteur effect, respiration and glycolysis of Ehrlich ascites carcinoma cells and their eventual death." Amygdalin at 10-30 mg/cc is said to produce "almost" no effects, unless of course glucosidase is added in which case an entire "death cycle" is produced in one or more hours. Other data appear in the full annual report of this project (NCI-331). Much of Dr. Burk's activities seem to be reported outside of the usual channels of scientific communication.

Serial No. NCI-375
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Homogeneity and structure of proteins

Previous Serial No.: Same

Principal Investigator: Elbert A. Peterson

Other Investigators: S. Ralph Himmelhoch and M. Clyde Otey

Cooperating Units: None

Man Years:
Total: 2 1/2
Professional: 1 1/2
Other: 1

Project Description:

Objectives: To develop methods and procedures for the separation and analysis of proteins and to apply these methods to the purification of specific proteins and the study of their structure-function relationships.

Methods Employed: In the work reported for this period, a variety of polyanions and polycations ranging widely in molecular size, charge density, and chemical composition have been prepared for use in the displacement chromatography of macromolecules. Most of these are ionic derivatives of low molecular weight dextrans, but other types are being explored. The polyanions have been evaluated as displacing agents, using test protein mixtures on columns of DEAE-cellulose. Effluent fractions were evaluated by electrophoresis in 33-place slabs of polyacrylamide gel, using procedures described in last year's report.

Major Findings: The feasibility of chromatographing proteins and other macromolecules of biological interest by displacement instead of elution has been established by experiments carried out on a semi-micro scale. Mixtures of polyanions having a suitable range of affinities for DEAE-cellulose have been shown to be capable of displacing and spacing serum proteins (used as a model mixture) to produce chromatographic fractions having unusually high protein concentrations. Since no increase in salt concentration or decrease in pH is needed, the operational aspects of chromatography are simplified: one merely applies the sample to the column and follows it with a selected mixture of polyanions. As with low molecular weight substances in other displacement systems, competition among the proteins and the polyanions (all

of which would be tightly adsorbed under the prevailing conditions of salt concentration and pH) causes them to become arranged in a series of contiguous zones moving down the column, their positions determined by their relative affinities. The spacing of the emerging proteins can be increased by using more polyanions of appropriate affinity, and the concentrations of the emerging zones are determined by the concentration of the most tightly bound polyanions in the displacing mixture.

The polyanions are readily separated from the protein fractions. The chromatographic fractions can be directly subjected to gel electrophoresis, without dialysis or concentration, since the high electrophoretic mobility of the polyanions carries these rapidly away from the proteins, leaving the latter in a milieu having a low conductivity. Thus, the examination of complex mixtures by a combination of chromatography and electrophoresis is greatly expedited, and such analyses become feasible on a semi-micro scale.

Significance: This kind of procedure, as yet in its early stages of development, promises to be of considerable value in the separation of proteins and nucleic acids at any scale of operation and therefore has significance to all research that involves the isolation of such substances. Improved resolution, more concentrated fractions, and greater convenience of operation can be expected as the potentials of displacement chromatography are more fully realized. Moreover, since migration is induced by displacers under conditions of salt and pH in which the migrating molecules are otherwise strongly adsorbed, the possibility arises of chromatographing materials that are "irreversibly" adsorbed in present procedures.

Proposed Course of Research: Studies of reaction conditions required to produce displacers having desired affinities will be continued in order to improve the distribution provided by present mixtures. The method, as it develops, will be applied to the separation of proteins in several readily available tissue extracts, to the subfractionation of t-RNA, and to the examination of proteins and nucleic acids in guinea pig bone marrow cells.

Honors and Awards: The Hillebrand Award for 1970 (to E. A. Peterson and H. A. Sober).

Publications:

1. Peterson, E. A.: Cellulosic Ion Exchangers. In Work, T. S. and Work, E. (Eds.): Laboratory Techniques in Biochemistry and Molecular Biology. Amsterdam, Netherlands, North-Holland Publishing Co., 1970, Vol. 2, pp. 225-400.

2. Hull, E. and Himmelhoch, S. R.: Purification of monkey α-fetoglobulin. J. Nat. Cancer Inst. In press

3. Himmelhoch, S. R.: Chromatography of proteins on ion-exchange adsorbents. In Jakoby, W. (Ed.): Methods in Enzymology. New York, Academic Press, 1970, Vol. 22, pp. 1181-1193.

4. Himmelhoch, S. R.: Leucine aminopeptidase: A zinc metalloenzyme. Arch. Biochem. Biophys. 134: 597-602, 1969.

Serial No. NCI-333
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

NIH-PHS
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical and immunochemical studies on normal and leukemic leukocytes.

Previous Serial No.: Same

Principal Investigators: Warren H. Evans, S. Ralph Himmelhoch, Michael G. Mage

Other Investigators: Elbert A. Peterson, Richard K. Desser, Charles Grieshaber, Thomas Gessner, Freesia Huang, Emma Shelton, Margaret Januska and Shirley Wilson.

Cooperating Units: Biosynthesis Section, Laboratory of Biochemistry, NCI

Man Years:
Total: 8 1/2
Professional: 8
Other: 1/2

Project Description:

Objectives: To investigate the biochemical factors regulating growth, maturation, and the phagocytic capacity of leukocytes, especially with regard to the arrested maturation and decreased phagocytic activity of leukocytes from myelogenous leukemia; to exploit any biochemical differences observed between normal and leukemic leukocytes for purposes of improving diagnosis and treatment of leukemia. The development of myeloperoxidase and alkaline phosphatase in the course of leukocyte maturation is being studied because of their favorable biological and physical properties. The chemical "structure" of the different classes of leukocyte granules is under investigation by chemical and immunological procedures.

Methods Employed: Leukocytes are isolated from bone marrow, blood, and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation and further fractionated by chromatography and electrophoresis. Immunological reagents, including fluorescent antibodies, are prepared from the purified fractions and used to follow the development of related material during maturation of the cells. The maturation of enriched cell populations is studied in soft agar and stationary tissue culture.

Major Findings: Cell culture conditions have been developed which permit the study of the maturation of bone marrow granulocytes *in vitro*. Quantitative studies of the absolute number of various cell types present in bone marrow

cultures at various times during a 96 hour period indicate that mature granulocytes increase 2-to 3-fold while immature granulocytes show a concomitant decrease in cell number. The mature cells formed in these cultures have a normal morphological appearance and appear to have normal levels of alkaline phosphatase and peroxidase as judged by histochemical staining. Studies on the synthesis of these enzymes are now underway and preliminary results of biochemical assays show that cellular alkaline phosphatase increase approximately 50% as granulocytes mature in vitro. No corresponding increase in neutrophil peroxidase has been detected thus far, using an immunochemical assay for this enzyme, presumably because of the low numbers of potential peroxidase synthesizing cells present in normal bone marrow. Immature granulocytes isolated by Ficoll sedimentation also mature in tissue culture, but the mature cells formed appear to be larger than normal and have hypersegmented nuclei. These mature cells also stain much more intensively for peroxidase than normal cells.

A search for factors in serum which might have possible regulatory effects on granulocyte maturation has been undertaken. Studies of normal guinea pig bone marrow incubated with RNA and DNA precursors (C^{14} formate, H^3-uridine, H^3-thymidine) in 24 hour stationary cultures indicate that dialyzed normal guinea pig serum stimulates RNA synthesis 3- to 4-fold and DNA synthesis 2-fold relative to dialyzed fetal calf serum controls. These effects were significantly greater in bone marrow suspensions in which the number of erythrocyte precursors was reduced 15-to 20-fold by hypertransfusion, suggesting that the serum factors might be specific for bone marrow leukocytes. Investigations are now underway to determine whether the synthesis of specific components of marrow granulocytes are affected by these stimulatory factors. Preliminary studies show that incorporation of C^{14}-labelled amino acids into the proteins of the granule fraction obtained from bone marrow granulocytes is stimulated 60% by normal guinea pig serum relative to controls utilizing fetal calf serum. A 100% stimulation above controls was obtained when serum from animals in which leukocyte production had been stimulated by leukapheresis was substituted for normal serum.

Studies on the subcellular distribution and possible functions of glycoproteins in granulocytes during maturation are being carried out. Evidence drawn from biochemical and radioautographic studies indicate that mature granulocytes are characterized by a high incorporation of H^3-glucosamine and a low incorporation of H^3-leucine into protein, whereas with immature granulocytes the opposite is true.

Preparations of pure eosinophil granules have been achieved by centrifugation of cells from eosinophil-rich exudates in albumin or Ficoll, followed by density gradient centrifugation of homogenates of the purified cells. The difficulties customarily encountered in obtaining soluble extracts of proteins from these granules have been overcome by the use of cationic detergents as extractants, and special gel electrophoresis systems permitting resolution of the granule proteins have been developed. Eosinophil granules yield only four visible bands, one of which has been identified as eosinophil peroxidase (CM-2). Of the several other enzyme activities that have been associated with the eosinophil granule, three (in addition to peroxidase) have been studied. Only two of these are detectable in purified granule fractions and neither coincides with

any of the four demonstrable protein bands observed on gel electrophoresis. The question whether these enzymes are granule constituents remains open.

Isolation of eosinophil peroxidase (CM-2) by previous techniques has been unsatisfactory because of the instability of the molecule and its strong non-ionic interactions with ion-exchange adsorbents. The use of CETAB as an extracting agent has permitted solubilization of the enzyme at low ionic strength where separation by usual techniques can be achieved. It has been discovered that ion-exchangers and gel filtration materials made of polyacrylamide permit much better chromatographic separation of this enzyme, with higher yields than formerly obtained. Using these materials, a convenient method for preparing large (~10 mg) quantities of physically and immunologically homogeneous CM-2 has been developed. The molecule has been shown to possess a heme different from that of CM-1 (neutrophil peroxidase) and tentatively, to have a molecular weight of 60,000-65,000.

The physical and chemical properties of purified human leukemic myeloperoxidases have been subjected to intensive study. Electron micrography and electrophoresis in gels containing sodium dodecyl sulfate demonstrate the enzyme to be made up of two similar subunits. Amino acid analysis has shown it to be particularly rich in arginine, lysine, glutamic acid, and aspartic acid, and present evidence indicates that it contains no cysteine. Metal analysis demonstrates the presence of 2 gram atoms of iron per mole of protein. Its molecular weight has been determined to be 130,000.

Significance to Cancer Research: Understanding of normal and leukemic maturation depends upon clarification of the molecular processes whereby the cell controls the appearance and disappearance of specific components. Such information has never been obtained in a systematic fashion similar to that achieved in morphological studies of normal and leukemic granulocytes.

Proposed Course of Research: We wish to establish as detailed an understanding of the nature and order of the biochemical events of maturation as has previously been acheived at the morphological level. A detailed description of the changes in the biochemical constituents of nuclei, cytoplasm, cell membrane, and granules at various important stages of white cell development will be attempted in order to identify possible sites of hormonal and genetic regulation in both normal and leukemic cells.

Honors and Awards: None

Publications:

1. Rosenblum, D., Himmelhoch, S. R., Peterson, E. A., Evans, W. H., and Mage, M. G.: Purification of alkaline phosphatase from guinea pig bone marrow. Arch Biochem. Biophys. 141: 303-312, 1970.

2. Evans, W. H., Wilson, C. M., Mage, M. G., Grieshaber, C. K., and Himmelhoch, S. R.: Biochemical characterization of isolated immature granulocytes from guinea pig bone marrow. J. Reticuloendothelial Soc. 9: 209-224, 1971.

3. Mage, M., Evans, W. H., Himmelhoch, S. R., and McHugh, L.: Immunological identification and quantification of guinea pig heterophil and eosinophil peroxidases. J. Reticuloendothelial Soc. 9: 201-208, 1971.

Serial No. NCI-5203
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of immunochemical and physical methods for the purification and characterization of immunocytes and their subcellular components.

Previous Serial No.: Same

Principal Investigator: Michael G. Mage

Other Investigators: Christiane Chersi

Man Years:
Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: The goal of this project is the development of methods for the isolation of immune cells from the nonimmune cells of lymphoid tissue, for the subfractionation of the various classes of immunocytes, and for their subcellular fractionation in order to study the mechanisms involved in the development of the different immunological activities of antibody proteins.

Methods Employed: Rabbits are immunized in the footpads to stimulate immunoglobulin production, and the popliteal lymph nodes are removed after labelling with radioactive amino acids and/or nucleosides. The tissue is disrupted and the polysomes are isolated by sedimentation in sucrose gradients. Anti-Ig antibodies used to precipitate polysomes are purified on immunoadsorbents.

Major Findings:
1) Nonspecific binding of soluble immunoglobulin to polysomes can be essentially eliminated when the polysomes are prepared in buffer containing 0.25 M KCl, instead of the more usual 0.025M to 0.1 M KCl.
2) Rabbit lymph node polysomes may be specifically precipitated by sheep antibodies to rabbit immunoglobulin, either by co-precipitation with carrier rabbit IgG, or by precipitating the sheep antibodies with antibody to sheep immunoglobulin. Interaction is presumably with nascent protein chains on the polysomes.

Significance to Bio-medical Research: A basic knowledge of cell growth and differentiation, including the development of immune reactivities, is needed to help in understanding infectious, degenerative, and neoplastic disease.

Such knowledge must be sought on the cellular and molecular level.

The specific precipitation of ribosomes acheived in this work is a significant step towards the isolation of immunoglobulin-mRNA for molecular studies of immunocyte differentiation and of the basis of antibody variability. Such studies may eventually aid in understanding the variation in tumor susceptibility of human beings and make possible the control of immune mechanisms of tumor rejection.

Proposed Course: We plan to improve our methodology for the separation of immunoglobulin-producing polysomes, to work on the isolation of their m-RNA, and on using this m-RNA in hybridization experiments.

Honors and Awards: None

Publications: None

Serial No. NCI-5202
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation, fractionation, and characterization of native nucleoproteins

Previous Serial No.: Same

Principal Investigator: O. Wesley McBride

Other Investigators: Harvey L. Ozer and Elbert A. Peterson

Cooperating Units: Biosynthesis Section, Laboratory of Biochemistry, NCI

Man Years:
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: Determination of the role of nucleic acid-bound proteins in the regulation of the transcription and translation of genetic information. Biological transformation of cells with isolated and fractionated native mammalian genetic material as a means of genetic mapping.

Methods Employed: Mammalian cells are grown in monolayer and suspension cultures and radioactively labelled with precursors of nucleic acid and protein. Cell lines lacking specific enzymes are isolated by growth of wild-type cells in selective media. Parasynchrony is induced in cultures by the addition of metabolic inhibitors or by selective detachment of mitotic cells. Metaphase chromosomes are isolated from synchronized cell cultures arrested at mitosis by inhibitors of microtubule formation. Differential and isopycnic centrifugation are used in the isolation of subcellular particles. Interphase nuclei and metaphase chromosomes are separated, and chromosomes further subfractionated, according to mass by unit gravity sedimentation. Chromosome fractionation is assessed by velocity sedimentation, phase and bright field microscopy, and volume distributions of particles. The molecular weight of cellular and isolated chromosomal DNA is determined by velocity sedimentation in alkaline sucrose density gradients. Chromosome uptake is measured by scintillation counting of cell lysates after incubation of cells with ^{3}H-thymidine-labelled chromosomes. Biological transformation is detected by colonial growth of transformed mutant (AZG-) cells in selective media after the ingestion of fractionated or unfractionated metaphase chromosomes isolated from wild type (AZG^{s}) Chinese hamster (CH) fibroblasts.

Major Findings: Procedures for induction of cell synchrony have been explored. The best method for isolating large numbers of mitotic CH fibroblasts (75-93% mitotic indices) is Colcemid arrest of cells following induction of parasynchrony in suspension cultures by the addition of excess thymidine to block DNA synthesis and reversal of the block with deoxycytidine. This increases the yield of metaphase chromosomes and reduces problems associated with removal of contaminant interphase nuclei and debris. The molecular weight of isolated chromosomal DNA is about 8-30 x 10^6 soon after isolation, but it decreases on storage at 5° C, probably because of the presence of contaminating nucleases. The molecular weight of DNA from all size classes of chromosomes is closely similar. A total lack of correlation between chromosome morphology and DNA molecular weight has been observed.

Unit gravity sedimentation provides significant fractionation of chromosomes according to mass. An electronic particle counter and pulse height analyzer was found applicable to analysis of chromosome sizes, and it provides an objective method for measuring the volume distribution of a statistically significant number of chromosomes in a brief time interval.

Cells can ingest chromosomes by phagocytosis. Results of microscopic and autoradiographic examinations designed to discriminate between intracellular uptake of chromosomes and simple physical binding to the surface of recipient cells are compatible with true uptake but are inconclusive. Chromosome uptake is time and temperature dependent, stimulated by serum and divalent cations, and greater on monolayers than in suspension. Polycations do not stimulate this uptake significantly. A large fraction of the ingested chromosomal DNA is degraded to low molecular weight ($< 10^6$) material within a few hours.

Cells are biologically transformed by ingested chromosomes with a relatively low frequency of about 10^{-6}, but this appears to be greater than the reversion rate of recipient cells in control cultures. Results obtained by exposure of cells to chromosomes that had been subfractionated according to size indicate that the Hypoxanthine-Guanine Pnosphoribosyl Transferase (HG PRTase) gene is probably located on a small CH chromosome.

Proposed Course of Study: Experiments that should distinguish between true biological transformants and revertants are in progress. Recipient cells containing other genetic markers will be isolated and studied. Stimulation of biological transformation will be evaluated using synchronized recipient cells at various stages of the cell cycle and employing chromosomes trapped in artificial lipid membranes to facilitate inactivated virus-induced fusion of chromosomes into the cell cytoplasm, thus circumventing lysosomal degradation of ingested chromosomes. Methods for isolation of better preparations of biologically active chromosomes are being investigated.

Significance: Metaphase chromosome isolation and fractionation is the most promising approach for the fractionation of mammalian genes and can provide material for the investigation of control elements associated with specific sets of genes. The biological expression of these chromosomes introduced into recipient cells can provide a method for mapping of mammalian genes. It could permit investigation of sites of integration of oncogenic viruses

into the mammalian genome, and differences between genetic elements in neoplastic and normal cells might also be detected.

Honors and Awards: None

Publications:

1. McBride, O. W. and Peterson, E. A.: Separation of nuclei representing different phases of the growth cycle from unsynchronized mammalian cell cultures. J. Cell Biol. 47: 132-139, 1970.

Serial No. NCI-380
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Deoxyribose containing compounds in normal, regenerating and cancer tissues.

Previous Serial Number: Same

Principal Investigator: Walter C. Schneider

Other Investigators: William Kidwell, Gilbert Smith, Pietro Gullino

Cooperating Units: Laboratory of Biology

Man Years:
Total: 3
Professional: 1
Other: 2

Project Description:

Objectives: The objectives of this project are to isolate and identify deoxyribose-containing compounds other than DNA from normal and tumor tissues, to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal and cancer tissues, to isolate DNA from subcellular structures other than nuclei, to study the physical and biochemical properties of extranuclear DNA and to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

Methods: Nuclei, mitochondria and microsomes were isolated from rat or mouse liver by differential and/or density gradient centrifugation. Nucleic acids were extracted from tissue fractions using sodium lauryl sulfate and phenol. DNA was purified from these extracts by treatment with ribonuclease and passage through Biogel P-60 or by banding in CsCl.

DNA samples were examined by means of analytical CsCl density gradient centrigugation, melting behavior, and amount of labeled thymidine incorporated. DNA was measured by the Burton modification of the diphenylamine reaction.

Major Findings: The experiments on the incorporation of labeled thymidine into lipids and proteins reported last year were completed and published.

The experiments carried out during the past year were concerned again with microsomal DNA. Specifically, attempts were made to establish whether this DNA could be differentiated from nuclear DNA because our previous work had indicated that microsomal DNA and nuclear DNA were identical in all respects except one: the rate at which thymidine was incorporated and the half life of the thymidine that had been incorporated.

In one experiment with Dr. Gullino, the hepatocarcinogen, 3'-methyl dimethylaminoazobenzene labeled with ^{3}H, was given to rats and nuclei, mitochondria, and microsomes were isolated from the liver the following day. Although all three fractions were heavily labeled, only about 1% of the radioactivity was present in the nucleate fractions isolated with phenol and less than 10 percent of this radioactivity was associated with DNA. Due to the low levels of activity no firm conclusions could be reached about the labeling of the nuclear, mitochondrial, or microsomal DNA.

In another experiment rats were given hydroxyurea prior to the ^{3}H-thymidine and the labeling of the DNA samples compared. The labeling of the mitochondrial DNA was the greatest and was depressed about 70 % by hydroxyurea. The labeling of the nuclear and microsomal DNA was inhibited even more, 95-96%, but the extent of the inhibition was the same in both types of DNA.

Since phenobarbital is known to induce the formation of microsomal enzymes, it was hoped that it might exert some specific effect on microsomal DNA. However, when the incorporation of ^{3}H-thymidine into nuclear, mitochondrial, and microsomal DNA was examined, it was found that incorporation was increased in all three types of DNA by phenobarbital, with the corporation into nuclear DNA being increased to the greatest extent.

Electron microscopic observations by Ender's et al. have indicated that luteal cells of the superovulated ovary contain large amounts of smooth endoplasmic reticulum. Such tissue would therefore appear to be a suitable object for the study of microsomal DNA and experiments along this line are being carried out with Drs. Kidwell and Smith.

Since portions of the endoplasmic reticulum have been observed to be closely associated with the outer nuclear membrane, experiments were carried out with Dr. Smith to determine if DNA resembling microsomal DNA was present in the outer nuclear membrane. While it was readily feasible to prepare intact nuclei and remove their outer membrane, the results were inconclusive because the nuclei were found to lose about one percent of their DNA each time they were washed. Consequently the DNA released when the outer nuclear membrane was removed could not be identified as coming solely from the membrane.

Significance: These experiments fail to establish the uniqueness of microsomal DNA and in fact the results with hydroxyurea add additional support for the idea that this DNA may be derived from the nucleus and adsorbed by the microsomes. Nonetheless, the microsomal DNA must represent a different type of DNA regardless of whether it comes originally from the nucleus or is a normal constituent of the microsomes. The function of this DNA can only be speculated upon. It might be considered to be a rapidly labeled nuclear DNA which is normally transported to the cytoplasm where it transmits its information and is then lost. Unfortunately, no evidence can be marshalled to support this view; a claim that certain tissue culture cells contained informosomes has not been confirmed.

The relationship of microsomal associated DNA to carcinogenesis has yet to be explored. The experiment described above in which a difference in binding of a carcinogen to DNA was sought was unsuccessful due to the low degree of incorporation of the carcinogen. This type of experiment should be carried out with other carcinogens and the turnover of the mitochondrial and microsomal DNA's should be studied in a variety of normal and tumor tissues.

Proposed Course: Experiments are still in progress on superovulated ovaries and on DNA-DNA hybridization which may shed some light on the intracellular origin of microsomal DNA. Studies on tissue cultures, on tumors, and on organs other than the liver will provide further information on both mitochondrial and on microsomal DNA.

Honors and Awards: None

Publications:

1. Schneider, W. C. and Greco, A. E.: Incorporation of pyrimidine deoxyribonucleosides into liver lipids and other components. Biochim Biophys Acta 228: 610-626, 1971.

2. Schneider, W. C.: Isotope dilution determinations and other studies of cytidine diphosphate choline, cytidine diphosphate ethanolamine, deoxycytidine diphosphate choline and deoxycytidine diphosphate ethanolamine in liver and liver tumor. J. Nat. Cancer Inst. 46: 435-441, 1971.

Serial No. NCI-5211
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Initiation and maturation phenomena in the replication of mammalian DNA

Previous Serial Number: NCI-5211

Principal Investigator: William R. Kidwell

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1-1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: The replication of DNA in a cell may be classified as two discrete processes, the initiation of replication and subsequently, chain elongation leading ultimately to mature, chromosomal sized DNA. Of these two processes, initiation (an event repeated as many as 10,000 times during one round of replication) is least understood. This investigation places emphasis on elucidating the mechanisms of initiation, the triggering event of replication.

Methods Employed: Cells were accumulated at the G-1/S boundary of the cell cycle by producing a thymidineless state with amethopterin. Subsequently, the S-phase was triggered by the addition of thymidine. Quantitative differences in satellite and nonsatellite DNA synthesis were assessed in intact cells or isolated nuclei at various stages of the S-phase. The two classes of DNA were resolved by means of isopycnic centrifugation or chromatographically, after denaturation and reannealing of the purified nuclear DNA. Synthesis of DNA was monitored by the incorporation of H^3-TTP (or H^3-thymidine in the case of whole cell experiments).

Major Findings: Discontinuous synthesis of satellite DNA during S. Nuclei of mouse L-cells contain a species of DNA, satellite DNA, which is readily resolvable from the bulk nuclear DNA. The timing of synthesis of satellite DNA was evaluated by pulse labelling cells with H^3-thymidine at various stages of S and subsequently fractionating the purified DNA. Such experiments revealed that satellite DNA was made both early and late in the DNA synthetic period with little or no synthesis of this species in the second quarter of

the S-phase. This pattern of replication was independent of the method of synchronization (amethopterin, cytosine arabinoside, fluorodeoxyuridine, or a double synchronization with excess thymidine). It appears that satellite DNA is not one but two or more independently replicating species whose buoyant densities and reannealing properties are very similar.

Fidelity of DNA replication in isolated nuclei. In an attempt to elucidate the mechanism of satellite DNA replication, an in vitro assay system consisting of nuclei isolated from S-phase cells was utilized. Replication in vitro appeared to be a continuation of the normal DNA synthetic process. Maximal incorporation of H^3-TTP into an alkaline stable, DNAse labile product required all from deoxynucleotide triphosphates and Mg^{+2}. Qualitative examination of the in vitro synthesized DNA revealed that satellite DNA was labelled maximally with nuclei from early or late S-phase cells. Mid S-phase nuclei synthesized satellite DNA at only 1/10th the level of early S-phase nuclei. Clearly the pattern of replication, the timing of synthesis of a particular kind of DNA was conserved in the subcellular system.

Differential amplification of satellite DNA synthesis. The soluble protein fraction of S-phase cells was shown to modify nuclear DNA synthesis in vitro. A standard preparation of nuclei from cells making little satellite DNA (Mid S) was tested with cytoplasmic extracts from early, mid and late S-phase cells. Both early and late S-phase cells extracts produced a selective stimulation of satellite compared to nonsatellite DNA synthesis (as much as 400% stimulation). Mid S-phase cytoplasm was without effect. The factors which preferentially stimulate satellite DNA synthesis were in highest concentration in cells whose satellite DNA synthesis was in progress (early and late in the replication period). The factor was heat labile and its appearance in the cytoplasm was blocked by treating the cell with actinomycin.

Significance to biomedical research and the program of the Institute. Generally increased rates of cell proliferation is a characteristic of malignancy. Central to the question of cell proliferation is an understanding of the factors governing DNA replication -- the initiation of synthesis and subsequent maturation into a second DNA complement. Elucidation of the controlling mechanism of DNA replication may suggest new targets for a chemotherapeutic approach to the cancer problem. Progress in this area is presently limited by the lack of specific assays for initiation. In this respect, the in vitro DNA synthesizing system which has been developed represents a significant advance in that the replication of a specific kind of DNA can now be examined.

Proposed Course:

The findings of the present study suggest a model for replication in which signals from the cytoplasm initiate DNA synthesis in a specific and ordered temporal sequence. Continued effort will be directed toward elucidating the nature of these signals. An attempt will be made to isolate the principles in early and late S-phase cytoplasm which preferentially stimulate satellite DNA synthesis in isolated nuclei.

Honors and Awards: None

Publications: None

Serial No. NCI-5205
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on biochemical mechanisms of sensitivity and resistance to antimetabolites and mechanisms for control of nucleic acid synthesis.

Previous Serial Number: Same

Principal Investigator: Elizabeth P. Anderson

Other Investigators: Thomas Q. Garvey, III, and Alec S. Liacouras

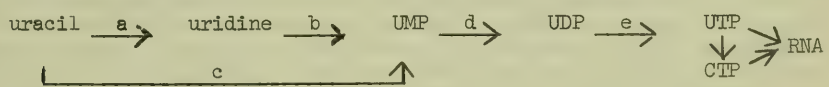
Cooperating Units: F. K. Millar, Laboratory of Physiology, NCI

Man Years:
Total: 4
Professional: 3
Other: 1

Project Description:

Objectives: The objective of this project is to investigate nucleotide metabolism in normal and rapidly growing cells, including neoplastic tissues. The aim is to elucidate pathways of biosynthesis, mechanisms of control over these pathways, and biochemical mechanisms of sensitivity and resistance to purine and pyrimidine analogs. The end-products of nucleotide biosynthesis are the triphosphate precursors of nucleic acids, and regulation of the enzymes involved in these biosyntheses is probably a major factor in determining the rate of cellular nucleic acid synthesis.

Methods: Work has been focused on the "salvage" pathways of pyrimidine nucleotide biosynthesis:



1. Studies on UMP-CMP Kinase.

Further studies have been carried out on UMP kinase (reaction d above) in Tetrahymena pyriformis, a rapidly growing protozoan unable to synthesize the pyrimidine ring de novo and hence dependent on the "salvage" pathways for its pyrimidine anabolism. The enzyme has been purified 300-fold from frozen-thawed lysates of Tetrahymena. The reactions catalyzed by the enzyme are:

$$\begin{matrix}\text{UMP} \\ \text{or} \\ \text{CMP}\end{matrix} + \text{ATP} \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \begin{matrix}\text{UDP} \\ \text{or} \\ \text{CDP}\end{matrix} + \text{ADP}$$

Initial velocity studies have been used to investigate the details and mechanism of the UMP kinase reaction; the reaction has been assayed as phosphorylation of C^{14}-UMP. The findings have ruled out a "ping-pong" mechanism, in which one of the reactants adds to, and is released from, the enzyme prior to interaction of the enzyme with a second reactant. This means that a ternary complex of the enzyme with both substrates is the central intermediate in the reaction. The apparent K_m's for the two substrates, ATP·Mg^{++} and UMP, have been found to be 3.0 mM and 1.0 mM respectively. The kinetics of the reaction with UMP and ATP·Mg^{++} were non-classical, and the pattern of the initial velocity plots suggested that two additions of the UMP substrate to the enzyme are possible. The active form of the ATP·Mg^{++} substrate appears to be a 1:1 complex of the nucleotide with Mg^{++}. This complex was also found to impose substrate inhibition at high levels if the ratio ATP·Mg^{++}/UMP exceeded 2. Further, the pattern of ATP·Mg^{++} inhibition of the reaction suggested proximity of interaction of the binding sites for the two substrates.

These studies have been carried out by Dr. Thomas Garvey. In addition to the biochemical studies, he has made use of the IBM 1620 computer for both routine calculations and for the more complicated problems of curve fitting and statistical evaluation of complex data. In these computer operations, he has collaborated with Dr. F. K. Millar, Laboratory of Physiology, NCI.

The results of these studies will be presented at the meetings of the American Society of Biological Chemists in San Francisco in June of this year.

2. Studies on Uridine Kinase.

Uridine kinase (reaction b above) is the initial anabolic step in the "salvage" pathway of pyrimidine nucleotide biosynthesis in mammalian tissues and has been shown to be rate-limiting and susceptible to feedback inhibition by nucleoside triphosphate end-products. This enzyme is being purified from acetone powder preparations of the mouse ascites mast cell neoplasm P815/S; so far a 40- to 50-fold purification has been achieved using fractionation on DEAE-cellulose, ammonium sulfate fractionation, and gel filtration on Sephadex. The enzyme has been assayed as conversion of C^{14}-labeled uridine to nucleotide product, and the partially purified preparation has been shown to be free of UMP and UDP kinases and thus of further phosphorylation of the product UMP. The apparent K_m of the partially purified enzyme for the substrate uridine was found to be 1.25 mM. Analyses have also shown that the sensitivity to feedback inhibition by CTP has been retained by the enzyme through these purification steps. These studies have been carried out by Mr. Alec Liacouras.

3. Studies on Uridine Phosphorylase.

Our studies have shown that fluorouracil- and fluorouridine-resistant sublines of the P815 mast cell neoplasm have markedly decreased levels of uridine kinase activity compared to the sensitive, "wild type" parent line. As a corollary of these findings, studies have been undertaken to compare the levels of uridine phosphorylase in the sensitive and resistant lines.

This enzyme is being assayed as the ribose-1-phosphate-dependent conversion of C^{14}-uracil to labeled uridine. In some other tumors, resistance to pyrimidine analogs has been associated with decreased levels of uridine phosphorylase as well as of uridine kinase. The initial evidence suggests that this may also be true in the P815 mast cell tumor lines.

Significance. Enzyme activities are direct and accessible sources of quantitative information about a cellular genome, and a deletion, addition, or alteration in the cell's "normal' genotype can frequently be deduced from the resulting alteration in the cell's "normal" complement of enzyme activities. For this reason, detailed studies on cellular enzymes, in terms of both enzyme function and the nature of the enzyme protein molecules, are a direct and sensitive method for probing subtle but significant differences between normal cells and abnormal ones (malignant, virally infected, etc.) in which the genome is suspected of being altered. This project explores by this method differences between normal and malignant cells and between cells sensitive and resistant to anticancer agents.

Proposed Course of Research: In the studies on UMP kinase, isotope exchange studies are now under way aimed at confirming, by an entirely independent approach, the conclusion that the reaction in not "ping-pong". This will establish directly whether or not one substrate can bind to the enzyme and release in the absence of the other substrate. It should also be possible to calculate the K_{eq} for the reaction from these data. Initial velocity studies and isotope exchange studies will also be made on the reaction of the enzyme with CMP in order to determine if the mechanism of phosphorylation is the same as that deduced for UMP. Product inhibition studies are also planned to establish whether the addition of substrates to the enzyme to form the ternary complex is random or occurs in an ordered fashion, and if ordered, what the order of addition is.

The purification of uridine kinase from the mast cell neoplasm will be continued through other steps and procedures until the level of purification makes it feasible to explore the molecular nature of the enzyme protein, particularly with regard to possible subunit structure. The mechansim of feedback inhibition will then be investigated in terms of the nature of the enzyme molecule.

Uridine phosphorylase will be explored further in the fluoropyrimidine-sensitive and -resistant lines of the 815 neoplasm, and the levels of activity will be quantitatively compared.

Serial No. NCI-397
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies comparing the structure, properties and metabolism of the nucleic acids and nucleoproteins of cultured cells and of tumors derived from them.

Previous Serial No.: Same

Principal Investigator: Jean Rotherham

Other Investigators: None

Cooperating Units: Tissue Culture Section, Laboratory of Biology, NCI

Man Years:
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To discover if there are changes in structure, properties or metabolism of the nucleic acids or nucleoproteins of cultured mammalian cells which are characteristic of and associated with transformation from pre- to post-malignant stages.

Methods and Major Findings: Some aspects of the role of vitamin B_{12} and folic acid in DNA-thymidine synthesis in a neoplastic murine cell strain, NCTC 5905.

We found that a function of vitamin B_{12} seems to be, in 5905 cells in culture, that of increasing DNA-thymidine synthesis by raising limiting levels of folate coenzymes that are required in the metabolism of "one-carbon fragments". Also some other suggested functions seems to be inoperative as judged by these results and those we have published previously.

Some additional data related to our previous studies concerning other aspects of the metabolism of nucleic acid precursors in cells of this strain were obtained.

These findings have been reported in a manuscript that has been submitted to the Journal of The National Cancer Institute but has not yet been accepted for publication.

Significance: The results described have contributed to a more complete understanding of some aspects of metabolic reactions by which these murine neoplastic cells synthesize DNA-precursors that are needed for continuous proliferation in culture.

Proposed Course of Work: This project has been interrupted. It is proposed that it be continued as soon as changes in and across organizational lines will permit the biological material to be available under workable conditions. Some of these changes are being made and others are still under consideration

Honors and Awards: None

Publications: None

Serial No. NCI-394
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on isozyme distribution in various transplantable rat hepatomas.

Previous Serial No.: Same

Principal Investigator: Theodore T. Otani

Other Investigators: Harold P. Morris

Cooperating Units: Cancer Research Unit, Department of Biochemistry, Howard University, College of Medicine, Washington, D.C.

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: Although a broader aim of the project is to study the role of isozymes of several enzymes on the physiology of the cell and to correlate this role to the problem of neoplasia, the aim of this portion of the project is to seek a relationship between activities and isozyme distribution of four enzymes and the growth rates of certain tumors.

Methods: The enzymes, aspartate aminotransferase (GOT), alanine aminotransferase (GPT), malic dehydrogenase (MDH) and lactic dehydrogenase (LDH) from normal rat liver and from transplantable rat hepatomas were studied. The hepatomas studied were 3924A, 5123tc, 7793, and R7. Their generation time ranges from 0.5 months for 3924A to 4.5 months for R7.

The enzyme assay was performed on a water extract of the tissues, which was prepared by (a) homogenization in a blender, (b) centrifugation to remove fragments, (c) dialysis and (d) further centrifugation to remove precipitated materials.

The enzyme assays for GOT, GPT, MDH and LDH are based on the measure of the rate of oxidation of NADH.

Isozyme distribution is determined by measuring enzyme activities of the buffer eluates of starch sections after electrophoresis in appropriate buffers.

Major Findings: Examination of specific activities and the isozyme distribution of these tumors indicated that (a) the activity of a given enzyme could be within normal range in one neoplastic state, and abnormal, either high or low, in another, and that the level of the enzyme activity does not necessarily

alter in one or another direction due to the neoplastic state, and (b) the relative distribution of isozymes of different enzymes varied considerably among tumors, but none resembled the distribution profile of the isozymes of the normal liver. That is, the enzyme patterns of the tumor tissues differed from the pattern of normal in the activities of at least one enzyme or isozyme. The enzyme patterns of Hepatoma 3924A, a fast growing, poorly differentiated tumor, exhibited the greatest deviation from those of the normal liver in that five of the six components of the enzyme profile were abnormal.

Significance: The enzymes studied here hold key positions in the pathways between amino acid and carbohydrate metabolism. There exist at least two biochemically distinct GOT and GPT isozymes in the rat liver, one primarily situated in the mitochondrial and the other in the supernatant fraction. There exist at least four isozymes of MDH in the normal liver and in the hepatoma, some similarly localized in the mitochondrial and others in the supernatant fraction. The occurence of at least five LDH isozymes is well known. It is possible that each isozyme by its location in the cell has a definite function, for example, in the case of GOT, whether to mediate conversion of α-ketoglutarate to glutamate, hence channeling substrates into the amino acid pathway, or to mediate conversion of glutamate to α-ketoglutarate, hence channeling substrates to energy production. In the normal cells, there is likely a delicate balance between these systems, perhaps, in some way influenced and controlled by hormones. Could one phase of a mechanism initiating neoplasia be due to, or cause a disruption of this balance? The findings from these studies, it is hoped, will contribute to the elucidation of this problem, as well as furnish some insight on the role and function of isozymes in the cells.

Proposed Course of Study: During these studies it was noted that the thermal decomposition behavior of certain isozymes was unconventional in that a revival of activity was observed after an initial normal deterioration. The study of this phenomenon will be pursued.

Honors and Awards: None

Publications: None.

Serial No. NCI-306
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on β-hydroxy-α-amino acids

Previous Serial No.: Same

Principal Investigator: Theodore T. Otani

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: To prepare β-hydroxy-α-amino acids and their derivatives by simple organic chemical methods, since some compounds of this class have been shown to possess some bacteriostatic as well as some anti-tumor actions.

More specifically, the objectives of this portion of the project are:
a) To prepare β-hydroxyhomomethionine and β-hydroxymethionine from glycine and methylmercaptopropionaldehyde and from glycine and methylmercaptoacetaldehyde, respectively.
b) To prepare various chain-halogenated β-hydroxy-α-amino acids from halo-alkyl aldehydes and glycine.
c) To prepare β-hydroxy-α-asparagine and β-hydroxy-β-asparagine from glycine amide and glyoxylic acid, and from glyoxylic acid amide and glycine respectively.

Methods: The preparation of these compounds is carried out in an alkaline medium with the appropriate aldehyde and glycine copper complex under conditions that are empirically determined.

Major Findings:

1. β-Hydroxyhomomethionine - Although favorable conditions for the formation of this compound have been determined previously, some difficulties were encountered in isolating the product in reasonable yields. Attempts have been made using diverse systems to increase the yield, but with no remarkable improvements. Some of the methods attempted were: a) Partition chromatography, using cellulose column, or extra thick filter paper, or a column of compressed circular filter paper, and various solvent systems to separate the product from

salts and from other ninhydrin positive materials of the reaction mixture. b) Separation of the product from the salts of the reaction mixture by selection of alcohol soluble base, such as lithium hydroxide, which would remain in solution while the amino acid is being precipitated. c) Separation of the product from the salts of the reaction mixture by precipitation of the salt by selection of a base such as barium hydroxide, which would produce a water insoluble compound upon gassing with carbon dioxide. d) Separation of the amino acid by selection precipitation from salts and other amino acids as the mercury salt. This method showed some promise in giving whiter products, but the yield was still no better than earlier methods.

The product obtained thus far, though in low percentage yield, and not completely white, has been shown to be chromatographically pure in four solvent systems.

2. The preparation of chain-halogenated β-hydroxyamino acids and of the β-hydroxyasparagines has been postponed until the preparation of β-hydroxyhomomethionine and of β-hydroxymethionine has been completed.

Significance: Some of the classes of chemotherapeutic agents now in use against cancer are hormones, nitrogen mustards, folic acid analogs, nucleic acid antagonists, and natural products. In examining these, it is interesting to note that the concepts of biological antagonism appears reasonable in the treatment of neoplastic disease, albeit, the point of attack by the compounds in the physiology of the system is crucial. For example, the folic acid analogs probably interfere metabolically as a coenzyme antagonist, and the halogenated pyrimidine derivatives, such as 5-fluorouracil, as substitute for the non-fluorinated natural pyrimidines in the synthesis of nucleic acids.

That amino acid metabolism may also be a susceptible area of action in tumor therapy was demonstrated by the efficacy of asparaginase in certain tumors which required asparagine. Hence, amino acid deprivation, either by the removal of the amino acid, as in the case with asparaginase, or by substitution with an analog, may be effective in inhibiting tumor growth.

The un-natural amino acids synthesized under this program are close structual analogs of essential natural amino acids, differing in some cases only in the presence of a single hydroxyl group adjacent to the α-amino group. Hence, β-hydroxynorleucine differs from norleucine only in the presence of a hydroxyl group in the β-position of carbon chain of the amino acid. β-Hydroxynorleucine is a structural analog of methionine, differing from the latter in having a methylene group in place of the sulfur atom in the γ-position of the chain. Indeed, β-hydroxynorleucine, even as a mixture of four isomers, not only exhibited inhibitory action in two microbial systems, but also showed some anti-tumor effect against carcinoma 755 as measured by the decrease of tumor size and increase in survival time.

β-Hydroxymethionine is an even closer analog of methionine than is β-hydroxynorleucine, differing from methionine only in possessing a β-hydroxyl group. β-Hydroxyhomoethionine is also an analog of methionine, differing from the latter in possessing one more methylene group than methionine, and a β-hydroxyl group.

The chain-halogenated amino acids, similarly, are close structural analogs of essential amino acids. For example, γ-dichlorothreonine would be an analog

of leucine, since the size of the chlorine atom is approximately that of the methyl group; also γ-triflurothreonine would be a close analog of threonine since the atomic radius of the fluorine atom is essentially that of hydrogen atoms.

Proposed Course of Study:
1. Further attempts will be made to increase the yield of β-hydroxyhomomethionine.
2. Effort will be made to increase the purity of β-hydroxyhomomethionine.
3. Chemical identification and proof of its structure will be made.
4. Its biological properties including anti-tumor effect will be studied, first on microbiological screening systems, then if found active in these systems, on mammalian systems.
5. Attempt to prepare β-hydroxymethionine, chain-halogenated β-hydroxy-amino acids, and the β-hydroxyasparagines will be continued.

Honors and Awards: None

Publications: None

Serial No. NCI-366
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

Project Title: Biosynthesis and assembly of intracellular structural components.

Previous Serial No.: Same

Principal Investigator: Edward L. Kuff

Other Investigators: Kira K. Luederz, Harvey L. Ozer, Nelson A. Wivel, Samuel H. Wilson, Dante Marciani

Cooperating Units: Laboratory of Cell Biology, NCI, NIH

Man Years:
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: To elucidate mechanisms by which the reduplication of complex intracellular elements is regulated and coordinated; to study synthesis and transport of individual macromolecules and their assembly into such complex structures; to study the effects of carcinogenic agents (viruses and chemicals) on these integrated functions.

Methods Employed: Cell fractionation by differential and density gradient centrifugation, isolation and gradient fractionation of nucleic acids and nucleoproteins, electron microscopy of isolated nucleoproteins, in vivo and in vitro incorporation of isotopically-labelled precursors into protein and nucleic acid, chromatographic, immunochemical and electrophoretic characterization of specific proteins.

Results: During the past year, work was continued on the structural and biological properties of intracisternal A-particles (see previous Annual Reports of this Project). These virus-like particles are found in a variety of mouse tumors, sometimes in great abundance, and also in normal cells of certain strains of mice. They are occasionally found in cells that are simultaneously producing mammary tumor virus (MTV) particles or, more commonly, C-type particles characteristic of murine leukemia virus (MuLV).

The particles were first isolated in this Laboratory from a transplantable plasma-cell tumor in BALB/c mice. Preparations contained 80% protein, 15% phospholipid and about 5% RNA (no DNA detected). The particles have now been prepared from four different transplant lines of plasma-cell tumors in BALB/c mice, one plasma-cell tumor of C3H mice, and from a neuroblastoma cell line of A/Jax origin. All show similar protein patterns upon electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. In each

case, a major proportion of the total protein is represented by a single component with molecular weight about 73,000. This component is contained within the inner particle shell, solibilization of which by SDS is critically dependent on disulfide bone reduction.

The protein components of intracisternal A-particles differ markedly from those of MuLV (Rauscher or Moloney strains) and MTV. A-particles are devoid of MuLV group specific antigen as judged by electrophoretic, complement fixation and immunodiffusion analyses. They are lacking in the interspecies antigen (gs3) of the RNA oncogenic viruses.

Immunologic tests specific for the major A-particle protein have now been developed using antisera prepared against particles from BALB/c myelomas. Under appropriate conditions, the antisera reveal a common antigenic determinant in a variety of mouse tumor cells known by electron microscopy to contain intracisternal A-particles, but fail to react with normal mouse cells, with isolated MuLV or MTV, or with cells that are producing MuLV but lack A-particles.

An extremely low level of apparent DNA polymerase activity has been detected in particles isolated from the BALB/c myeloma cells. Studies of template specificity are in progress.

Significance to Cancer Research: Intracisternal A-particles may represent the expression of a viral genome in the affected cells, perhaps related to the neoplastic properties of the cells. It is important therefore to determine their relationship to known oncogenic RNA viruses and to determine the prevalence of expression of A-particle antigens in mouse tumor cells. In addition, we believe these particles may be useful in studying processes of assembly and budding common to many membrane-enclosed viruses.

Proposed Course of Research: Structure, synthesis and assembly of the intracisternal A-particles will be investigated. Concomitantly, we will study the intracellular localization of synthesis of other specific proteins and the transport and assembly of these proteins into more complex cellular structures.

Honors and Awards: None

Publications: None

Serial No. NCI-5210
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Control of specific protein synthesis in animal cells.

Previous Serial No.: Same

Principal Investigator: E. Brad Thompson

Other Investivators: S. R. Levisohn, J. V. Miller, Jr. and D. Aviv

Cooperating Units: Dr. S. S. Yang, Bionetics Research Labs; Dr. P. Cutrecasas, Dept. of Medicine and Pharmacology, Johns Hopkins Univ; Dr. V. Manganiello, M.D., NHI, NIH, Bethesda, Md., Dr. A. Gilman, LBG, NHI, NIH, Bethesda, Md., Dr. H. Sober, LNE, NIAMD; Dr. D. Nebert, LBS, NICH, NIH; Dr. Higuchi, Microbiological Associates; Dr. G. Williams, BGY, NCI, NIH, Bethesda, Md., Dr. F. K. Miller, LPHY, NCI, NIH, Bethesda, Md.

Man Years:
Total: 5
Professional: 4
Other: 1

Project Description:

Objective: The overall objective is to discover and define the mechanisms by which normal and malignant cells control gene expression. An important subordinate object is to determine the mechanism of action of steroid and other hormones in inducing hepatic enzymes.

Methods Employed: We are using conventional methods of tissue culture, spectrophotometric and radioactive enzyme assay, radioimmunoprecipitation, cell fusion, chromatography, radioautography, etc. We have developed a method of affinity chromatography which can be used to purify ribosomes synthesizing tyrosine aminotransferase, as well as the enzyme itself. Much of our work is possible because of HTC cells, a rat hepatoma tissue culture line we developed several years ago. In these cells, tyrosine aminotransferase is induced, as in liver, by certain steroid hormones.

Major Findings:

1) Cell-free protein synthesis: An initial study of tyrosine aminotransferase synthesis from ribosomes partly purified for this activity through affinity chromatography has allowed synthesis of measurable amounts of enzyme in a cell-free system. This work is now in press. An important implication of the purification process is that nascent transaminase chains may be able to fold sufficiently to bind with high affinity to their cofactor. We have begun to study, using this system, possible control steps in the synthesis of the enzyme.

Work on the purification of TAT by affinity chromatography is nearly complete and should be ready to submit for publication in the next few months.

To facilitate these studies we have been working with Dr. H. Sober to develop efficient, economical techniques for the large scale growth of HTC cells.

2) Several projects on cellular genetics involving variants from the "wild-type" HTC cell line are in progress.

a) One such clonal variant, HTC-S, is constitutive for the enzyme TAT, responding only very slightly to steroids. The HTC-S line has been characterized biochemically in work being readied for publication. We hope by contrasting HTC-S with wild-type cells, to discover site(s) of control of enzyme synthesis.

b) A study of the rate and range of phenotypic variation in HTC cells is underway. We have developed a histochemical stain for TAT and shown (in work now in press) that essentially all wild-type HTC cells contain the enzyme. Now using unselected clones, we are studying the range of variation in the wild poopulation and are following the natural rates at which selected high- and low-inducing clones develop variations.

c) A variety of drug-resistance markers have been developed in the HTC and other cell lines to provide tools for future genetic experiments. The markers include BUdR, 8-azaguanine and actinomycin D resistance. Also, tyrosine protrotrophs have been derived.

d) HTC cells have been adapted to growth in serum-free medium, without loss of enzyme inducibility. It is rare for a cell line which possesses a differentiated function to be found in serum-free medium.

3) Studies on tRNA in HTC cells have been continued. We have found that phenylalaninyl tRNA increases after steroid treatment, with kinetics similar to those of enzyme induction. It is our intention to work out the mechanism of the increase in tRNA and to find the relation, if any, between the tRNA and the enzyme increase.

4) Various cell fusion studies between inducible and non-inducible cells have been carried out.

a) It has been shown that non-inducibility is rapidly dominant to inducibility when HTC cells are fused with TAT-negative cell line derived from the same inbred animals.

b) Various clones of hybrid cells have been grown out, utilizing selective media and our durg-resistant cell lines. In hybrids between L-cells (TAT non-inducible, adenyl cylase-positive, non-contact inhibited) and HTC cells (TAT inducible, very low adlenyl cyclase, non-contact inhibited) we found that TAT induction was absent, adenyl cyclase was at the higher, L-cell levels, and there was contact inhibition. Other fusion experiments are at earlier stages of work-up.

Significance to Cancer Research: This work promises to be of value in understanding how normal and malignant cells control the expression of their genetic material. The hybrid experiments suggest that if certain specific functions can be shut off when cells are hybridized, there must be specific controlling molecules involved. The formation of a contact-inhibited line from two non-contact-inhibited parents offers an excellent model for studying the difference between regulated and unregulated growth. We are trying to design experiments which would reveal the gene(s) carrying the information for expression of contact inhibition.

The studies on the non-inducible variant of HTC cells, on the inducible tRNA of HTC cells and on cell-free synthesis of TAT are all aimed at elucidating the molecular site of action of steroid hormones and the specific sites of regulation of gene expression and protein synthesis in these cells. We believe that these goals are worthy of the effort.

Honors and Awards: None

Publications:

1. Thompson, E. B., Granner, D. K., Tomkins, G. M.: Superinduction of tyrosine aminotransferase by actinomycin D in rat hepatoma cells. J. Molec. Biol. 54: 159-175, 1970.

2. Thompson, E. B.: Biochemistry of the lipids and proteins of white matter. In Vinker, P. J. and Bruyn, G. W. (Eds.): Handbook of Clinical Neurology. Amsterdam, Netherlands, North-Holland Publishing Co., 1970, Vol. 9, pp. 1-22.

3. Thompson, E. B., Levisohn, S. R. and Miller, J. V. Jr.: Steroid control of tyrosine aminotransferase in hepatoma tissue culture (HTC) cells. Proceedings, III International Congress on Hormonal Steroids, Hamburg, Germany, 1970. In press.

4. Thompson, E. B. and Tomkins, G. M.: A histochemical method for the demonstration of tyrosine aminotransferase in tissue culture cells, and studies on this enzyme in HTC cells. J. Cell Biol. In press.

5. Miller, J. V., Jr., Cuatrecasas, P. and Thompson, E. B.: Partial purification by affinity chromatography of tyrosine aminotransferase-synthesizing ribosomes from hepatoma tissue culture cells. Proc. Nat. Acad. Sci. (USA). In press.

Serial No. NCI-379
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural characterization of immunoglobulins, enzymes and other biomacromolecules.

Previous Serial No.: Same

Principal Investigator: Emma Shelton

Other Investigators: (Immunoglobulin structure) R. T. Acton, J. C. Bennett, A. M. Smith, P. F. Weinheimer; (Complement structure) R. M. Stroud, K. Yonemasu; (Glutamine synthetase structure) T. Duell.

Cooperating Units: Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama Medical Center, Birmingham, Alabama; Division of Biology, California Institute of Technology, Pasadena, California; Laboratory of Cell Biology, NCI; Laboratory of Biochemistry, National Heart and Lung Institute.

Man Years:
Total: 4
Professional: 3
Other: 1

Project Description:

Objectives: To relate the fine structure of macromolecules to their biochemical and physical properties, and to their function in biological systems.

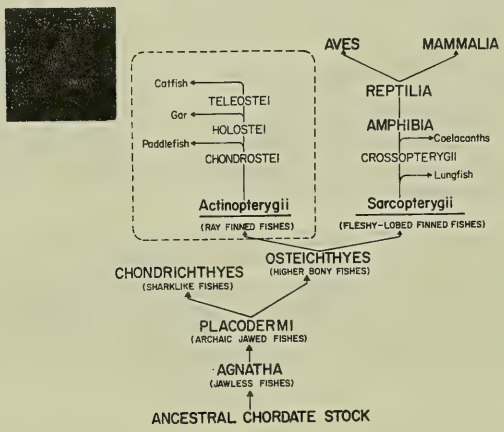
Major Findings:

a) Phylogenetic relationships of immunoglobulin strcutre

During the past year, in collaboration with immunochemists at NIH and at the University of Alabama Medical Center, we have been able to show that the IgM-like immune macroglobulins of certain fishes display tetrameric radial symmetry. These immune macroglobulins, isolated from three orders of the subclass Actinopterygii, were found to have a sedimentation co-efficient of 14S, a molecular weight of ~600,000 and a carbohydrate residue distribution different from human macroglobulins. The physical-chemical characteristics of these macroglobulins were very different from those isolated from man, rabbit mouse, sheep, chicken and dogfish and considerable confusion as to their exact nature prevailed until it was demonstrated with the electron microscope

that the molecule was made up of four subunits instead of the usual five.

The class Osteichthyes is divided, on anatomical grounds, into two major subclasses, Sarcopterygii and Actinopterygii (see below). The subclass Actinopterygii has often been considered an offshoot from the main evolutionary line leading to mammals. The finding that all members of this subclass produce unique tetrameric immunoglobulins, strengthens their classification as a group of fishes branching from the main stem of the evolutionary tree, and provides a basis for tracing changes in gene pools during evolution.



b) Structure of the complement component, C1q.

The complement system consists of nine components or eleven distinct serum proteins. The first component of complement, C1, is composed of three proteins, C1q, C1r and C1s. The primary target of the of the complement system is the cell membrane, and the complement component C1q appears to be responsible for the binding of complement to the cell membrane on the one hand and to specific antibody on the other.

Purified C1q, M.W. ~360,000 is a very labile molecule containing an unknown number of subunits. The molecular structure C1q is unknown but progress toward solving its ultrastructural configuration has been made during the past year. We have not yet been able to convince ourselves that we have looked at intact C1q molecules with the electron microscope. What we see appear to be disrupted molecules, thus it is only possible to say at the present time that the whole C1q molecule is made up of an undetermined number of dense subunits which measure, on the average, 65-70 Å in diameter. These subunits are joined at a central attachment area by strands estimated to be 60-100 Å in length and 15-20 Å in width. The strucutre of the central attachment area is hard to define, but is definitely less complicated than the central region of the IgM molecule. The subunits usually display themselves in a circle about the central area but not infrequently they can be seen to cluster together in a semicircle to one side of the central area. The diameter of the

whole molecule is 300-400 Å.

c) Structure of B. subtilis glutamine synthetase

The ultrastructure of glutamine synthetase derived from E. coli was thoroughly described by Valentine (Biochemistry 7: 2143, 1968), but the enzyme isolated from B. subtilis displayed such different physical-chemical properties from the E. coli enzyme that the question was raised as to whether the arrangement of the subunits in the macromolecule were also different. We could detect no morphological dissimilarities between the E. coli enzyme and the B. subtilis enzyme.

Proposed Course of Study: To elucidate further the structure of immunoglobulins and complement by examination of the fine structure of their subunits. To study conformational changes in (a) immunoglobulins upon interaction with antigen and (b) complement upon interaction with immunoglobulins.

Importance to Medical Research: A knowledge of the structure of macromolecules obtained by observations with the electron microscope provides in many cases an unequivocal basis for a knowledge of their function as well as an unequivocal confirmation of physical-chemical data. Thus it was possible to show that certain fish macroglobulins with molecular weights substantially lower than human macroglobulins were made up of four rather than five subunits. The exploration of the structure of complement components may lead to an insight into its interaction with membranes and immunoglobulins.

Honors and Awards: Chairman, Session on Macromolecules: Enzymes and Structural Proteins; 7th International Congress for Electron Microscopy, Grenoble France.

Guest Lecturer, Dept. of Immunology and Rheumatology, University of Alabama Medical Center, Birmingham, Ala.

Invited Speaker, 4th Complement Workshop, Johns Hopkins University Medical School, Baltimore, Md.

Publications:

1. Pollard, T. D., Shelton, E., Weihing, R. R. and Korn, E. D.: Ultrastructure characterization of F-actin isolated from Acanthamoeba castellanii and identification of cytoplasmic filaments as F-actin by reaction with rabbit heavy meromyosin. J. Molec. Biol. 50: 83-90, 1970.

2. Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E. and Stadtman, E. R.: Bacillus subtilus glutamine synthetase. Purification and physical characterization. J. Biol. Chem. 245: 5195-5205, 1970.

3. Shelton, E., Lueders, K. K., Kuff, E. L.: Observations on nucleoprotein with high specific radioactivity released from ^{3}H-uridine labeled polyribosomes. Proceedings of the Seventh International Congress for Electron Microscopy (Grenoble) France, p. 593-594, 1970.

Serial No. NCI-5215
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biological and ultrastructural studies on cell cooperation in the immune response.

Previous Serial No.: None

Principal Investigator: Emma Shelton

Other Investigators: M. Mage

Cooperating Units: Protein Chemistry Section, Laboratory of Biochemistry, NCI

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: To examine the interaction of lymphocytes and macrophages cooperating in the immune response in order to answer questions relating to:
a) the role of the macrophage in the immune response and
b) the type of lymphocyte responding to various antigens i.e., lymphocyte heterogeneity and
c) localization of antigen and antibody in cooperating cells.

Major Findings:

a) Setting-up a model system which will permit biological and ultrastructural examination of cell cooperation in the immune response.

The diffusion chamber constructed with cellulose membrane filters and implanted by laparotomy in mice represents a culture system that permits long-term culture of macrophages and lymphocytes (Shelton and Rice, Amer. J. Anat. 105: 281-341, 1959). Cells growing in the chamber can readily be labeled by intraperitioneal injection of radioisotopes and can be treated as a tissue for examination by light or electron microscopy.

During the past year we have cultured peritoneal cells from "normal" mice and from mice immunized by the intravenous injection of 10^8 sheep erythrocytes (SRBC). We have tested the ability of such cells to produce hemagglutinating antibody with the following results:

1) Cells from normal mice grown in presence of SRBC produced no detectable antibody.
2) Cells from immune mice grown in absence of SRBC produced

no detectable antibody.

3) Cells from immune mice grown in presence of SRBC produced high titers of hemagglutinating antibody. Antibody production started between 3 and 6 days and continued for at least 20 days. As few as 12,000 peritoneal cells grew and produced high antibody titers.

4) The morphology of the cells was directly correlated with agglutinin production. Presenting SRBC to "immune" cells provoked the formation of large numbers of plasma cells, many of which were clustered near Macrophages.

b) Search for proper antigen

For the background on this section, see the 1969 Annual Report. Progress in this area during the past year is as follows:

1) Explored possibility of using the hapten trinitrophenyl (TNP) to localize antibody in cells. Theoretically, anti-TNP antibody produced in response to TNP-bovine gamma globulin (BGG) could be localized by incubating anti-TNP-BGG-producing cells with TNP-labeled horseradish peroxidase (HRP). Project was abandoned when it was found that HRP would not bind TNP. The lysine residues to which TNP would theoretically attach are buried in the molecule and are not available for reaction. We are now exploring ovalbumin, M.W. 44,000 as an antigen with the possibility of using microperoxidase, M.W. 1900 (Feder, N., J. of Histochem. Cytochem. 18: 911, 1970) as an ultrastructural label.

Importance to Medical Research: An examination at the ultrastructural level of cells interacting in the immune response should lead to an expansion of our knowledge of how such cooperation is effected. An understanding of such cooperation has ultimate usefulness in the treatment of cancer and other diseases.

Proposed Course of Study: By using tracers visible at the ultrastructural level, we hope to follow the interaction of macrophages and lymphocytes during the production of antibody in the secondary response of these cells while growing in diffusion chambers. By separating and recombining macrophages and lymphocytes, we hope to visualize ways in which the macrophage participates in the immune response.

Honors and Awards: Member, Editorial Advisory Board, Cancer Research
Member, Specialty Fellowship Board, National Cancer Institute, 1970.

Publications:

1. Shelton, E., Daves, S. and Hemmer, R.: Quantitation of strain BALB/c mouse peritoneal cells. Science 168: 1232-1234, 1970.

Serial No. NCI-5212
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Virus-cell interaction of oncogenic viruses

Previous Serial No.: Same

Principal Investigator: Harvey L. Ozer

Other Investigators: Robert Wittes, Peter Tegtmeyer (Not NCI)

Cooperating Units: Dept. of Pharmacology and Microbiology, Case-Western Reserve Medical School, Cleveland, Ohio

Man Years:
Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: To study the interaction of oncogenic viruses, particularly SV 40, with mammalian cells, utilizing conditional lethal (temperature-sensitive) mutants of SV 40 and cells.

Methods Employed: Routine virological, immunological, and tissue culture techniques. Chemical mutagenesis of virus and/or cells. Analysis of amino acid radiolabelled infected cell extracts on CsCl and sucrose density gradients; analysis of viral structural proteins by acrylamide gel electrophoresis.

Major Findings:

1) Synthesis and assembly of SV 40.

The polypeptide composition of intact virions and empty shells has been determined on acrylamide gels. Both are composed of a single major capsid protein of 45,000 daltons (75-85% of total protein). Intact virions also contain 3 low molecular weight polypeptides (13-25,000), but they are much reduced in empty shells. Pulse-chase experiments with radioactive lysine indicate that the two types of viral particles are synthesized at different rates in infected monkey cells. The results of combined immunological and biochemical studies are consistant with a soluble pool of capsid protein which is incorporated rapidly into particles during maximal virus synthesis but slowly at the early stages of virus assembly. Despite the persistent synthesis of cellular proteins during infection, as much as 10% of the total protein

synthesized in a two hour pulse may be devoted to the major capsid protein.

2) Analysis of temperature-sensitive mutants of SV 40.

In collaboration with Dr. P. Tegtmeyer, we have been characterizing the first large series of SV 40 mutants. Three classes have been identified at the non-permissive temperature: 1) unable to synthesize viral DNA or capsid protein; 2) synthesizes viral DNA and capsid protein but unable to assembly viral particles; 3) assembles unstable viral particles resulting in accumulation of empty shells. By genetic analysis, the first group is distinct from the latter two. Comparison of mutant and wild-type infected cells (see Section 1) have permitted the identification of two non-virion forms of the major capsid protein: membrane-associated capsid and capsomeres of capsid protein. Transformation studies with these mutants are in progress by Dr. Tegtmeyer.

3) Isolation of temperature-sensitive mutants in the mouse cell line, BALB-3T3.

BALB-3T3 has been extensively studied as a model system for viral transformation in tissue culture. These cells are susceptible to both DNA (SV 40) and RNA (MLV, MSV) tumor viruses. In addition, they can be killed by productive infection with polyoma virus (biochemically similar to SV 40). Studies are in progress to select conditional lethal mutants in 3T3 defective in DNA synthesis. Such mutants should be valuable in evaluating the role of different cellular functions in transformation by and replication of oncogenic viruses.

Experiments to date have permitted development of procedures for mutagenesis of cells, selective killing of DNA-synthesizing cells by radioactive thymidine, and assay of viral and cell DNA synthesis.

Significance to Cancer Research: Understanding of SV 40 replication and the types of control operative in the synthesis of its structural proteins may provide insight about the repression of these proteins in virus-induced tumor cells. The genetic approach of isolating conditional lethal mutants of both virus and cells has been valuable in microbial biochemistry and has only recently been applied to animal cells and their viruses. Inasmuch as viral transformation appears to involve persistence of the viral genome in the tumor cells, probably through integration into the cellular DNA, study of cell mutants defective in DNA synthesis should permit analysis of the mechanism of viral integration.

Proposed Course of Research:

1) Continue studies on synthesis of viral structural proteins and assembly of viral particles with special emphasis on viral mutants and procedures which selectively effect viral versus cell protein synthesis.

2) Isolate and characterize temperature-sensitive mutants of 3T3 cells. The mutants will be selected on the basis of failure to incorporate lethal amounts of radioactive thymidine into cell DNA at the non-permissive

temperature. Mutants will then be screened to select those with defects in functions directly involved in DNA synthesis (initiation, polymerization, etc.). Polyoma virus infection will be utilized to help in localizing the block in DNA synthesis. Mutants will then be used in analyzing processes of transformation by SV 40 or RNA tumor virus.

Honors and Awards: Invited speaker at annual convention of National Science Teachers Association, March 29, 1971, on "Current Concepts in Cancer Research."

Publications:

1. Shah, K. V., Goverdhan, M. K. and Ozer, H. L.: Neutralizing antibodies to SV 40 in human sera from South India: Search for additional hosts of SV 40. Amer. J. Epid. In press.

Serial No. NCI- 5213
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Sept. 1, 1970 through June 30, 1971

Project Title: Radioassay techniques for gas producing reactions

Previous Serial No.: None

Principal Investigator: Samuel H. Wilson

Other Investigators: M. N. Kronick, NIH, Division of Research Services

Cooperating Units:

Man Years:
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objectives: To develop procedures for rapidly and efficiently measuring the release of radioactive gases from chemical reactions. Particular emphasis has been placed on the development of a rapid reproducible method for measuring release of $^{14}CO_2$ from enzyme reactions. Currently available techniques for measuring release of $^{14}CO_2$ are inconvenient and time consuming. An additional disadvantage is that determination of reactions rates are difficult to perform on single reaction mixtures.

Methods Employed: Mammalian brain L-glutamic acid decarboxylase activity was used as a standard CO_2 producing enzyme for development of new assay techniques. Various methods for measuring $^{14}CO_2$ have been investigated including liquid scintillation counting, ionization chamber counting and gas-solid scintillation counting.

Major Findings: A new technique has been developed for the radioassay of gas producing reactions. The new technique is simple and rapid and may be applied to the assay gas producing reactions on a routine basis. The technique employs a closed system reaction device consisting of a glass-reaction well and a hollow tent-shaped piece of scintillator plastic. Radioactive gases evolved during reactions are contained in the scintillator plastic tent and are evaluated using commercially available liquid scintillation counters. The time course of chemical reactions may be followed using a single reaction mixture.

Significance to Cancer Research: The new assay technique could prove to be a valuable analytical tool, not only for the assay of enzyme reactions but also for a wide variety of gas-producing chemicals reactions. One of the most promising applications of the new assay technique is in the continous and rapid assay of gas production by biological material such as cells in culture.

Proposed Course of Research: Current experiments directed toward perfection of the assay device will be continued. The gas assay technique will be applied to the detection of $^{14}CO_2$ released from cells growing in monolayer and suspension culture.

Honors and Awards: None

Publications:

1. Wilson, S. H., and Kronick, M. N.: A new assay technique for reactions that produce radioactive gases. Anal. Biochem. In press 1971.

Serial No. NCI-5214
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Sept. 1, 1970 through June 30, 1971

Project Title: Mechanism and regulation of RNA synthesis in eucaryotic cells.

Previous Serial No.: None

Principal Investigator: Samuel H. Wilson

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1/4
Professional: 1/4
Other:

Project Description:

Objectives: To understand the major aspects of the mechanism of RNA synthesis in eucaryotic cells. The production of ribosomal RNA in the yeast Saccharomces cerevisiae will be used as a model system during the initial phase of the study.

Great strides have recently been made in our understanding of the nature and structure of genes, major aspects of the mechanism of transfer of information from genes to protein, and the rules by which newly formed proteins assume specific tertiary and quarterinary structure and thence function. This new understanding has come about largely through the use of the two general approaches: (1) the isolation of small portions of complicated processes and their use in well-defined in vitro systems representative of the more complex in vivo state and (2) the use of cells and viruses that could be genetically manipulated.

One of the areas in biology yet to be understood even in a broad sense, is the mechanism of regulation of cellular growth in eucaryotes including differentiation and malignant transformation. It is the aim of the present research to apply the two approaches mentioned above to a study of growth regulation in eucaryotic cells. RNA synthesis has been chosen because RNA is fundamentally involved in cellular information transfer processes and because ribosomal RNA synthesis generally parallels growth rate in most eucaryotic cells. Mechanisms of regulation of transcription in eucaryotic cells may involve a significant qualitative jump in complexity and organization relative to transcription in bacteria. The search for new transcription mechanisms universal to all eucaryotic cells is one of the goals behind this

research. Yeast is being used as a model system in these studies because it possesses many unique advantages. The macromolecular constitution of the organism is much more like animal cells than the simpler bacterial systems. Yet, the genome of yeast is only 4 times larger than the genome of E. coli. Genes in yeast are distributed among 18 linkage groups or chromosomes and their location on these linkage groups may be assessed using easily performed segregation studies. By using the more simple eucaryotic organism such as yeast, one may have an improved chance of clearly defining transcirption mechanisms and discovering new principles of regulation that are quantitatively different from those in bacteria.

Methods Employed: The mechanism of transcription will be probed using a dual approach. Well-defined in vitro systems for the synthesis of ribosomal RNA will be developed and the catalytic and structural proteins involved will be assessed using temperature-sensitive mutants defective in ribosomal RNA production.

Proposed Course of Research: Since this is the initial report of the Project, I would like to review in some detail my proposed method of approach.

1) To isolate the genes for ribosomal RNA in various degrees of purity.

Genes for ribosomal RNA will be isolated from an aneuploid strain of Sacchromyces cerevisiae that carries 2X the normal amount of ribosomal genes. Ribosomal genes will be purified from isolated DNA by cesium sulfate centrifugation and differential hybridization.

2) To develop in vitro systems for synthesis of ribosomal RNA using purified ribosomal genes and RNA polymerase from E. coli.

Ribosomal genes in various stages of purity (from the purification procedure outlined above) will be used as template with RNA polymerase purified from E. coli strain A-19, lacking in ribonuclease I. Reaction mixtures will be assayed for ribosomal RNA synthesis by RNA:DNA hybridization, ion-exchange chromatography and RNA fingerprint analysis. The assay for in vitro synthesis of RNA will be highly specific because only ribosomal RNA will be detected. Techniques will also be developed for rapid, routine assay of reactions for ribosomal RNA synthesis using ion-exchange chromatography.

3) To determine the location and nature of promoter regions for RNA polymerase along the ribosomal genome.

Initiation of transcription may be controlled by the presence of one or more regulator units preceeding ribosomal RNA genes to be transcribed. Investigation of possible regulatory regions in the genes will be the major subject of emphasis during the first phase of this project. The existence of such regulatory regions will be probed by first determining the minimal size of the gene unit necessary for fidelity of transcription of ribosomal RNA. The minimal transcription unit will be isolated and then further sheared into smaller pieces, regulation regions will be detected by binding with RNA polymerase, using a Millipore filter binding assay.

4) To apply RNA polymerase isolated from yeast to the in vitro RNA synthesis system, outlined above.

Roeder has shown that yeast, like other eucaryotic cells studied to date, contains three detectable activities of DNA-dependent RNA polymerase. RNA polymerase I is resistanct to the inhibitor α-Amanitin and appears to be responsible for the transcription of ribosomal RNA. Using the in vitro R-RNA synthesis assay system RNA polymerase I from yeast will be isolated and the conditions for its transcriptase activity will be determined.

5) To elucidate, using in vitro systems, the protein and other factors important in ribosomal RNA poduction.

As outlined above under 2, dependence on low molecular weight, protein and nucleic acid factors will be probed, using the in vitro system described. The initial phase of the project will be conducted with E. coli protein factors including core DNA-dependent RNA polymerase, sigma factor, psi factor and rho factor. E. coli factors will be used for this initial phase because of their availability and because the primary objective of the initial phase of the work will be to characterize the structural and functional aspects of the gene itself and not protein factors. It will be of interest to learn whether protein factors from E. coli will transcribe ribosomal RNA genes from yeast and from coli equally well.

6) To select temperature-sensitive mutants defective in ribosomal RNA synthesis.

It will be possible to gain information about the number of protein factors essential for ribosomal RNA production and control using temperature-sensitive mutants defective in ribosomal RNA synthesis. Such a genetic approach is attractive because it is possible using the intact complex cell, to determine the number of protein factors important in ribosomal RNA synthesis. Such information is an essential guide in developing the in vitro R-RNA synthesis system mentioned above. Temperature-sensitive mutants of a haploid strain of Sacchromyces cerivisiae containing no auxotrophic markers will be selected using conventional procedures in growth medium supplemented with several independent sugar, nitrogen and vitamin sources.

7) To determine whether temperature-sensitive defects in ribosomal RNA synthesis are nuclear in origin, dominant or recessive with respect to the wild type, and if they are single gene mutations.

Analysis of temperature-sensitive mutants will be performed using conventional techniques of yeast genetics.

8) To study, using the in vitro RNA synthetic system outlined above, protein factors isolated from cells with temperature-sensitive defects.

The protein factors defective in temperature-sensitive cells will be identified by reconstituting the in vitro RNA synthetic system with individual factors isolated from temperature-sensitive mutants. This phase of the study awaits development of an in vitro synthetic system that is dependent upon protein factors from yeast, and is feasible only after the factors present in wild type cells have been identified and at least partially characterized.

9) To determine the linkage group and the map location of the ribosomal RNA genes.

This portion of the project will be conducted in collaboration with Dr. G. R. Fink of Cornell University. The approach we will use to determine the location of the ribosomal RNA genes in yeast will be similar to the approached used recently by Atwood and coworkers to determine the location of the ribosomal RNA genes in E. coli. Various aneuploid strains of Sacchromyces cerivisiae will be assayed for their content of ribosomal RNA genes using RNA-DNA hybridization.

10) To classify the temperature-sensitive mutants into groups based on their complementation patterns.

Complementation analysis can be rapidly and easily performed with yeast by mating haploid cells of opposite mating type containing defects in ribosomal RNA synthesis and testing the resultant diploid for the presence of ribosomal RNA synthesis at the restrictive temperature. Since the aim of this portion of the study is to determine the number of individual complementing groups important in ribosomal RNA synthesis, complementation analysis will be performed on a sufficient number of haploid mutants so that we may be satisfied that most of the parameters important in ribosomal RNA synthesis have been identified, at least genetically.

11) To determine the linear arrangement of the 140 ribosomal RNA genes in haploid yeast.

The physical linkage between the units of transcription of 16s and 27s ribosomal RNA, silent spacer regions, will be investigated by fragmenting ribosomal DNA in various states of purity followed by hybridization with purified ribosomal RNA.

Major Findings: None

Significance to Cancer Research: An understanding of the mechanism and regulation of RNA synthesis is one of the major unsolved problems in biology. The primary significance of using yeast as a model system for the study of RNA transcription is that it is perhaps the only eucaryotic system that is readily amenable to genetic as well as biochemical approaches. Analysis of transcription of a specific gene in yeast, such as the ribosomal RNA gene will lead to basic information about transcription, as well as techniques necessary for experimental approaches to the more complicated eucaryotic systems.

Honors and Awards: None

Publications: None

Serial No. NCI-368
1. Laboratory of Biochemistry
2. Tumor-Physiopathology Section
3. Bethesda, Maryland

PHS-NIH
Indivudual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the mechanisms of mammary tumor regression.

Previous Serial No.: Same

Principal Investigator: Pietro M. Gullino

Other Investigators: F. H. Grantham, B. Berghoffer, I. Losonczy

Cooperating Units: None

Man Years:
Total: 5
Professional: 1
Other: 4

Project Description:

Objective: To understand what is happening in the neoplastic tissue when a mammary tumor is regressing.

Methods Employed: Primary 7-12 DMBA and transplantable MTW9 rat mammary tumors were used as models. Both were regressing when deprived of the appropriate hormone stimulation. Some basic physiopathologic characteristics of the regressed tumors were defined first and then the ability of the regressing tumor to utilize substrates was measured as an approach to detect the early sequence of events occurring at the beginning of regression.

Major Findings:
1) A difference in the histologic picture between growing and regressing tumors did not appear until the tumor had lost at least 1/2 of the initial volume or more. These changes are of degenerative nature and in the cell compartment. The number of macrophages in a tumor regressed to 1/2 the initial size was not appreciably increased as compared with the growing tissue. Incorporation of carbon particles and histochemical demonstration of acid phosphatase were used to define the phagocytes. The regression process appears to start without any obvious change either in the gross morphology or in the type of cells of the tumor.
2) Tumors regressed to about 1/2 the initial size maintained rougly the same gross chemical composition of the growing tumors when the content of DNA, RNA, total protein, phosphoprotein, triglycerides, phospholipids and water was determined using established methods. The results were expressed per g of tumor or DNA content. At any time during regression the tumor resumed growth

when the proper hormonal stimulation was resorted. The gross composition of the regrowing tumor was similar to that of the regressing tumor.

The DNA content per nucleus in tumors regressed to 1/2 the initial size was similar to that of growing tumors (8.5-10 pg). The same was true for the ratios between nuclear DNA and cytoplasmic protein.

3) Regressing tumors had a vascular space equal to or larger than that of a growing tumor and the same was found for the blood flow as measured per unit weight in vivo. The efficiency of the vascular system of a regressing tumor appears to be as good as that of a growing tumor. The efficiency was evaluated directly by comparing the changes in tumor flow produced by altering pressure and blood volume of the host, and indirectly by measuring the ratios of available and utilized oxygen at various levels of blood flow. There was no indication that tumor regression started or progressed because of inefficiency of the vascular system.

4) The in vivo consumptions of oxygen and glucose were equal to or larger in regressing than in growing tumors. The values were expressed per unit weight of the tissue or DNA content. The regression process requires consumption of energy.

5) In pulse labeling experiments the capacity to incorporate uridine-$5H^3$ into RNA and glycine-$2C^{14}$ into protein and lipids was about 3 fold less in tumors regressed to 1/2 the initial size as compared with growing tumors. On the contary, during the first hours of hormonal deprivation the incorporation increased sharply. At peak incorporation the RNA:DNA ratios also increased.

When uniformly labeled glucose C^{14} was continuously infused in the aorta of the host at a dose less than 0.1 percent of the amount consumed by the tumor, so that changes in the glucose pool of the tumor were avoided, it was observed that:

a) the specific activity of RNA, protein, and lipids extracted from the nuclear and cytoplasmic fractions increased 2-3 fold during the first 24 hours of regression as compared with growing tumors treated in the same way.

b) these specific activities were decreased 4-5 fold in the regressing as compared with the growing tumor treated simultaneously when glucose infusion was continued until the regressing tumor had reached 1/2 the initial volume.

Regression of hormone dependent tumors appears to be a coordinated sequence of events started in and controlled by the neoplastic cells. Regression requires consumption of energy and, at the onset, synthesis of RNA, protein, and probably lipids.

Significance: The work contributes to the understanding of the conditions for growth arrest and regression of experimental mammary tumors. Since the experimental models mimick some of the properties of human breast cancer, this work contributes to the understanding of the human desease.

Proposed Course: To continue the study of the mechanism of mammary tumor regression. In particular to ascertain the importance of estrophilic protein in the regression process.

Honors and Awards: None

Publications:

1. Gullino, P. M.: Techniques for the study of tumor physiopathology. In Busch, H. (Ed.): Methods in Cancer Research. New York, New York, Academic Press, 1970, Vol. 5, pp. 45-91.

2. Watters, C. and Gullino, P. M.: Translocation of DNA from the vascular into the nuclear compartment of solid mammary tumors. Cancer Research In press.

Serial No. NCI- 5216
1. Laboratory of Biochemistry
2. Tumor-Physiopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Role of lysosomal enzymes in the regression process of mammary tumors.

Previous Serial No.:

Principal Investigators: Yoon Sang Cho-Chung and Richard Lanzerotti

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 3
Professional: 2
Other: 1

Project Description:

Objective: To ascertain whether the increment of activity of lysomal enzymes during tumor regression is due to a new formation of protein and to assess the function of these enzymes in the regressing process.

Methods Employed: Primary 7-12 DMBA and transplanted MTW9 mammary tumors were used. Both tumors regressed when the host was deprived of the proper hormonal stimulation. Six lysosomal enzymes, acid phophatase, arylsulphatase, β-galactosidase, β-glucuronidase, cathepsin and acid ribonuclease, were compared in growing and regressing tumors. The activities were measured in a saline homogenate with and without disruption of lysosomes by Triton X-100.

Major Findings:
1) The activity of all six enzymes increased during regression of both tumors. At six hours after hormonal deprivation acid ribonuclease was increased already 5-6 fold in the saline homogenate and 2-3 fold in the saline and Triton X-100 homogenate. The other 5 enzymes increased as ribonuclease but only at 48 hours after castration.
2) The increment of enzymatic activities was mostly confined to the supernatant of saline homogenates (free activity) rather than Triton X-100 treated homogenates (lysosomal bound activity).
3) Actinomycin D blocked the increase of free enzymatic activity, suggesting that this increment may be due to new formation of enzymes.
4) Puromycin also inhibited the increase of "free" lysosomal enzyme activity, again suggesting that this increase was the result of protein synthesis. The

half lives of the "free" forms of all enzymes as measured by the use of puromycin were found to be shorter than that of bound forms.
5) To confirm the above data, purification of ribonuclease and β-glucuronidase is in progress.

Significance: The work contributes to the understanding of the regression process in experimental tumors which mimick some properties of human breast cancer.

Proposed Course: To continue the study of the mechanism of mammary tumor regression. In particular to interfer with the action of lysosomal enzymes in order to assess whether their increased activity causes the destruction of tissues or is a secondary effect which follows a degradation controlled by other factors.

Honors and Awards: None

Publications: None

Serial No. NCI-334
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The occurrence and function of a stress-modifiable insulin: anti-insulin hormonal complex regulating glucose utilization in cancer and normal cells, and its relation to chemotherapy at normal and hyperthermic temperatures.

Previous Serial No.: Same

Principal Investigators: Mark W. Woods and Dean Burk

Other Investigators: None

Cooperating Units: Dr. Katherine K. Sanford, Tissue Culture Section, Laboratory of Biology, NCI; Dr. Walter Heston, Chief, Laboratory of Biology, NCI; Mr. George Vlahakis, Laboratory of Biology, NCI.

Man Years:
Total: 3 3/4
Professional: 3
Other: 3/4

Project Description:

Objectives: To determine the extent to which insulin:anti-insulin hormonal regulation of glucose metabolism functions as a metabolic control system in cancer and normal mammalian cells, and to determine ways in which this system can be manipulated as an adjunct to in vivo therapies (e.g., chemotherapy and hyperthermia) including the role of such regulation in host defensive cells (RES).

Methods: The responses of tumor and normal tissues to anti-insulin inhibitors of glucolysis such as steroids, compounds with steroid-like action (e.g., delta-1-testololactone, diethylstilbestrol, Podophyllum derivatives), and to substances which counteract this inhibition (e.g., insulin and bacterial endotoxins) are determined. Since glucolysis in cells which have a strong anti-insulin:insulin regulation of glucolysis show an increased K_m (low affinity) for glucose, and conversely, cells with weak anti-insulin regulation a low K_m (higher affinity) for glucose, comparison of the rates of acid production ($Q N_2CO_2$) at widely different glucose levels (e.g., 0.28% vs. 2.8%) constitutes a direct and accurate method for assaying the degree of regulation operative. The proper comparative levels of glucose to be employed will depend on the histological types of cells under study. We have used these techniques

in comparing the glycolytic properties of a large number of primary and transplanted liver tumors and primary and transplanted mammary tumors.

Major Findings: From studies in diverse types of normal tissues, e.g., kidney, brain, spleen, liver, etc. and different types of tumors, e.g., melanomas, mammary carcinomas, tissue culture-originated sarcomas, lymphocytic and granulocytic leukemias, hepatomas etc., it is clear that essentially the same type of steroid-insulin control regulates the rate of glucolysis in all of the tissues, both normal and malignant although tissue-specific quantitative differences do occur (e.g., with respect to glucose affinities). In all cases where the relevant non-malignant cells of origin have been available for comparison the derived tumor cells have shown not only increases in rates of anaerobic glucolysis, but probably more important, decreases in the susceptibility of glucolysis to inhibition by anti-insulin steroids. Similar progressive decreases in susceptibility of glucolysis to such hormonal regulation were found in tumor spectra based on progressive anaplasia (e.g., S91 malanoma derivatives and Sanford tissue culture tumor clones).

Normal liver parenchyma cells show a distinctive anaerobic glycolytic pattern in which acid formation proceeds from endogenous non-glucose substrates (glycogenolysis) and from both endogenously and exogenously supplied glucose (glucolysis). In collaboration with Mr. George Vlahakis we have extended our matabolic analyses of "spontaneous" primary hepatomas, adjacent tumor-free liver, livers of tumor-free mice, mammary tumors (primary and transplanted) etc. Mouse strains included C_3H, C_3HfB, C_3HA^{vy}, $C_3HA^{vy}fB$, BALB/c and C57 BL (mammary tumors only). The following transplanted hepatomas were studied (all identified on histological and metabolic grounds as belonging to the liver parenchyma family). The following transplantable liver tumors have been established by Mr. George Vlahakis; No. 12747 (thru 11 generations), No. 16650 (1-2 generations), No. 16824 (1st generation), No. 16832 (1-3rd generations) and No. 18303 (1-2 generation). At the present time the examined tissues can be arranged in a "spectrum" based on the Q N_2CO_2 values obtained at 0.28% glucose and ratio of increase with 10-fold higher glucose. The following approximate ratings have been experimentally obtained to date. (In all cases tissue samples were suspended in Krebs-Ringer medium plus 0.1% sodium pyruvate and oxygenated for 15 minutes before measuring the glycolytic rates anaerobically over a 120 minute incubation period): NORMAL LIVER (Q N_2CO_2 at 0.28% glucose = 0.2, increased 7-fold at 2.8% glucose); HOST LIVER adjacent to primary hepatomas (0.3, increased 5X); PRIMARY HEPATOMAS (1.0, increased 3X); transplanted hepatoma 12747 (gen. 6-11) 1, increased 6X); t.p. hepatoma 16650 (1-2 gen.) 1.5, increased 3X; t.p. hepatoma 16824 (1st gen.) 1.5, increased 3X; t.p. hepatoma 16832 (1-3 gen.) 2, increased 2X; t.p. hepatoma 18303 (1st gen.) 3, increased 2X; and CHOLANGIOMA (2-3 gen.) 5, increased 1.3X. Measurements of glycolysis in a large number of primary hepatomas have shown that significant increases in glucolysis almost always characterize the tumors as compared with adjacent non-tumor liver tissues. The only apparent exceptions have been certain large primary hepatomas in old C_3H male mice (21 months). The glucolytic profile of such tumors appeared strongly "normalized" e.g., tumor Q N_2CO_2 values at 0.28% glucose = 0.30 with 4.8 fold increase at 2.8% glucose.

The respective host liver values were 0.32, increase 4.4X. The ontogeny of these "normalized" tumors is not known, but glucolytic "hot spots" (probably small concealed primary tumors embedded in apparently normal liver) were occasionally encountered (Q = 3.0 at 2.8% glucose) indicating that highly active tumors can also exist in these mice. The ratios of the rates of glucolysis at low and high glucose levels, although subject to some variability, are nevertheless quite characteristic for each of the tissues studied. While the rate of tumor growth also depends on a number of other factors, the glucolytic capacity (measured anaerobically in vitro) appears to be critically related to tumor growth potentials in the intact animal and may be a major point of control. The new data obtained with the C_3H mouse liver tumors show a considerable range in glucolytic capacities which appear to be perpetuated in asexual cell inheritance.

Importance to Cancer Research: An increase in glucolytic potential appears to be one of the fundamental changes involved in the conversion of normal cells into malignant tumors. The present use of mouse strains developed by Heston and Vlahakis with high incidences of "spontaneous" hepatomas has made possible a better understanding of the nature of these glucolytic changes associated with cancer development.

Proposed Course: Studies are underway in which the abilities of particular transplanted hepatomas (e.g., 12747 vs. 18303) to grow in hosts of differing genetic constitution and sex will be compared. The response to anti-glucolytic steroids and/or other agents will be compared in mice held at normal and stressful temperatures. Current studies of the effects of exogenously added insulin and/or cyclic-AMP on metabolism in various cell types will be continued. In addition to tumor and normal cell lines currently under study other cell preparations (tissue cultures, marrow separations, clinical materials, etc.) will be examined metabolically as they become available. The occurrence of progressive resistance to steroid therapy as occurs in human prostatic and mammary carcinomas suggests the need for better understanding of the possible glycolytic roles of steroids in directly or indirectly regulating growth of such neoplasms.

Honors and Awards: None

Publications:

1. Sanford, K. K., Barker, B. E., Parshad, R., Westfall, B. B., Woods, M. W., Jackson, J. L., King, D. R. and Peppers, E. V.: Neoplastic conversion in vitro of mouse cells: Cytologic, chromosomal, enzymatic, glycolytic and growth properties. J. Nat. Cancer Inst. 45: 1071-1085, 1970.

Serial No. NCI-330
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Production of healthier cigarettes.

Previous Serial No.: NCI-330 (plus 1965-1966 NCI-331 and 332)

Principal Investigator: Dean Burk

Other Investigators: None

Cooperating Units: D. D. Tiggelbeck, Pittsburgh Activated Carbon Division, Pittsburgh; G. H. Cassels-Smith, J. S. Young Company, Baltimore, Md.; Vincent Lisanti, Council for Tobacco Research U.S.A., New York City, and P. and R. Grimm, Delcron Co., New York City.

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: The development and application of cell metabolic techniques for identification and biological quantitation of harmful components in cigarette smoke, and the development of effective filters and air dilution devices for eliminating these components.

Methods Employed: Tumor and other cell materials are exposed in vitro to various types and quantities of smoke, and effects on cell metabolism, growth, or death determined, manometrically, microscopically, and by staining. Manometrically one measures effects on fermentation, respiration, and the Pasteur Effect, with tumor, normal mammalian cells, or yeast.

Major Findings: Our new, rapid, fundamental, biological test system for harmful cigarette smoke (Proc. Amer. Assoc. Cancer Res., 10, 11, 1969; 11, 13, 1970) has been applied to a study of effects of air dilution of cigarette smoke on various experimentally measured biochemical and biological indicator end-points of smoke harm. The air dilutions of smoke were carried out by the most effective device we have found to produce accurate and graduated dilutions of smoke, with short-distance-and-time delivery to Ehrlich ascites carcinoma cells maintained in Krebs-Ringer-glucose-bicarbonate medium in manometric vessels. This device, the Delcron Count-Down dial cigarette holder, by means of graduated holes, mechanically dilutes smoke with air to yield smoke at relative smoke:air

ratios of 0.5, 0.4, 0.3, 0.2, 0.1 and 0, at corresponding dial settings of 100, 80, 60, 40, 20 and 0, with unchanged pressure drops (draw) at all settings, as we have determined with the new Phipps and Bird Single Port Smoking Machine (and also their older Falling Water Machine) attached to a Warburg manometer as pressure gauge. At any fixed low to moderate volume of smoke:air mixtures, and with appropriate manometry, the aerobic glycolysis of the cancer cells increased proportionally to the dial setting, at rates substantially independent of time and established within minutes. High and excessive volumes of smoke:air mixtures yielded progressive (time-dependent) inhibitions of glycolysis, and progressive cell death as measured by intracellular staining of nucleic acids with Trypan Blue or intracellular proteins with eosin, and by altered cell morphology. The glycolysis measurements were recorded automatically with Mecrolab instrumentation attached to the Aminco manometric apparatus. The biological tests measure the effects of both the particles ("tar") and the gas phases of smokes, either separately or combined, as desired.

The Count-Down Holder provides the smoker with a means of cutting down the smoke puffed in per cigarette or pack by as much as he wishes or may adapt to, even without reducing the number of cigarettes smoked. For instance, using the holder setting at 20, the net smoke effect for a pack-a-day smoker would be equivalent to smoking only 2 cigarettes without the holder; and, similarly, for a setting at 40, only 4 cigarettes without the holder, per day; etc.

Proposed Future Investigations: Development of combined use of holder with most effective filters.

Significance to Cancer Research: The Count-Down "dial" cigarette holder is, in my laboratory testing experience, the most effective mechanical device on the market for positively reducing smoking hazards, including carcinogenesis and where appropriately possible should also be studied, and supported by NCI, NIH and HEW.

Honors and Awards: Dean Burk, listed in Who's Who in the World, Vol. I, 1971.

Publications: None in common conventional form.

Serial No. NCI-331
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Anticancer effects of Amygdalin and derivatives

Previous Serial No.: Same

Principal Investigator: Dean Burk

Other Investigators: A. R. L. McNaughton, McNaughton Foundation of California, Sausalito, Calif.; Dr. Hans Nieper, Sibersee Clinic, Hanover Germany; Forschungsinstitut Manfred von Ardenne, Dresden, Germany

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: The development and application of cell metabolic techniques for identification and biological quantitation of harmful components of amygdalin and some of its chemical derivatives.

Methods: Tumor and other cell materials are exposed in vitro and in vivo to various amygdalin type compounds and effects on metabolism, growth, or death determined, manometrically, microscopically, and by staining. Manometrically one measures effects on fermentation, respiration, and the Pasteur Effect, with tumor and normal mammalian cells or with yeast.

Major Findings: For any given concentration of amygdalin, prunasin, mandelonitrile, and HCH-benzaldehyde, the aerobic glycolysis of Ehrlich ascites carcinoma cells maintained in Krebs-Ringer-bicarbonate-glucose medium in manometric flasks at 37° C is increased increasingly in the order of compound named, and the increases observed occur within minutes after compound addition, and may be maintained for hours, unless the increases observed approach the upper level of anaerobic glycolysis (Pasteur Effect approaches zero), in which case metabolic death ensues (decreases of aerobic glycolysis and respiration to zero), followed in due course by stainability of the cells with Trypan Blue (or eosin), and by other morphologic alterations (blebbing and cell enlargement). Ten-30 mg/cc amygdalin produces almost no effects even after many, many hours, unless, of course, the enzyme glucosidase is also added, in which case the entire death cycle is produced in one or more hours, due to prompt liberation of HCN and benzaldehyde. Ten-30 mg/cc prunasin produces a 50-100% increase in

aerobic glycolysis (but not to the full level of anaerobic glycolysis) that is sustained for many hours, but eventually the death phenomena ensue); initial addition of glucosidase produces results similar to those when glucosidase is added to amygdalin, for the same reason. Ten micrograms/cc mandelonitrile (in sesame oil) produces effects equivalent to those of 10 mg/cc mandelonitrile (i.e., is some 1000 times more potent an inhibitor), and higher concentrations produce prompt cell death, due to synergistic effects of HCN and benzaldehyde liberated spontaneously in aqueous media, without added glucosidase. Up to 1 mg/mandelontrile/cc carrier oid, given intraperitoneal by several times a week did not produce death of mice but 2 to 3 mg did.

Significance To Cancer Research and Proposed Future Investigations: To be studied, especially re spontaneous tumors in mice and (elsewhere) in man.

Honors and Awards: Dean Burk listed in Who Who's in the World, Vol. I, 1971.

Publications: None in common conventional form.

PHS-NIH
Summary Report
July 1, 1970 through June 30, 1971
Laboratory of Biology
NCI

During the year the Staff of the Laboratory of Biology has published 20 papers. Some of the findings are summarized here.

Several of the Staff, including Doctors Stromberg, Gantt, and Litwack, have been collaboratively working with the Avian Myeloblastosis Virus (AMV) as a model for studying the structure and biochemistry of a representative C-type RNA tumor virus. Dr. Stromberg has developed a rapid, simple, and quantitative method of separating, by the use of an appropriate surfactant, the envelope of the AMV virion from the core. This now enables him to characterize what is host contributed and what is virus-specified. When the envelope is removed ATPase activity is obliterated, RNA dependent DNA polymerase specific activity is increased 6-fold identifying this enzyme as a core constituent, and group specific antigenicity is maintained, establishing the core location of the Gs antigen.

Dr. Gantt has found that a specific RNA methylase is associated with the AMV and also with the Rous sarcoma virus. This is important in that it adds to the evidence implicating aberrant methylation of nucleic acids as a primary chemical event in certain malignant transformations.

Dr. Litwack is now attempting to localize in the core or noncore constituent of AMV 4S transfer RNA that has been reported in intact AMV. This is of interest in determining the role of tRNA in the virus life cycle and addresses the broader question of the mechanism of virus assembly.

Equal emphasis is being given to the more complex mouse mammary tumor virus (MTV) with its subline NIV that we have shown is closely tied to the host genome. It has been shown that in our strain C3H-A^{vy}fB NIV produces an incidence of mammary tumors of 90 percent although it is not transferred as MTV through the milk but more as a genetic factor through the male as readily as through the female. The A^{vy} gene is particularly influential in stepping up the virulence of NIV or making the mice more susceptible to its oncogenic activity.

Dr. Smith has been interested in the intracisternal A particle and comparison of it with MTV. He and Dr. Wivel have found that these A particles are ubiquitous and are vertically transmitted. They have found them in tissues derived from all three germ layers. They have isolated the cores of MTV and have found that they share no common antigens with the intracisternal A particles. Dr. Gantt is extending his methylase studies to MTV and other murine oncogenic viruses. In their immunologic studies involving the production of enzyme-labelled antibodies for the identification and localization of antigenic sites in cells Doctors Hoffman, Smith, and Wivel have demonstrated

that peroxidase-labelled anti-MTV antibodies will specifically stain budding and mature MTV virions and that they are active in immunologic tests specific for MTV antigens.

Because of our interest in inducing mammary tumors with pituitary isografts in strains of mice without MTV, we have given C57BL/M females pituitary isografts and have induced tumors in approximately 50 percent of them. There has been no evidence that C57BL/M mice have the mammary tumor virus in any form.

The staff of the Tissue Culture Section have continued their study of the effect of different kinds of serum in the media on bringing about the neoplastic transformation. It has been shown that fetal calf serum inhibits the neoplastic transformation. In his studies of fractions of the serum Gantt has shown that the factor in serum that causes the spontaneous malignant conversion is in the small molecule fraction.

A new observation on the serum is that mouse cells in mare serum do not undergo neoplastic transformation even after long periods of cultivation, while cells in stallion, gelding, and fetal horse serum do undergo malignant transformation.

Two methods of predetermining malignancy of mouse cells in vitro have been determined; 1) toxicity of 7,12 DMBA to long term neoplastic lines, the non-neoplastic lines are more resistant; and 2) certain cytological criteria developed by Sanford and Barker.

Numerous new formations of chemically defined media with no serum or protein have been tried for starting fresh cultures, but none has been found to be significantly superior to medium NCTC 135 that has been in use for some time.

In his methylation studies Gantt has shown that both strands of satellite DNA are equally methylated and that when the specific activity of main band DNA is compared with the specific activity of satellite DNA in the paired neoplastic and nonneoplastic cell lines there is no difference in methylation.

Dr. Hoffman has continued his genetic studies of mouse urinary proteins and catalase. The components of the urinary protein complex for both the 1a and 1b genetic types have been isolated by ion exchange chromatography. The carbohydrate composition of the mouse urinary protein has been determined and only one component of the complex has been found to be a glycoprotein. There are differences in peptide mapping between the components from the two genetic types. Linkage studies of catalase have shown that the Ce gene reducing catalase in certain mouse strains is probably on a different chromosome from the radiation induced acatalasemia mutant, C^{b}_{s}. It now appears that the Ce locus may contain a multiple allelic series with at least 3 alleles regulating the turnover of the enzyme.

In the continued work on instrumentation in tissue culture two highly controlled constant temperature rooms have been designed, installed, and tested. A portable self-contained constant temperature box on wheels with its own power unit has also been designed in collaboration with the manufacturer

and has been tested and found to be a highly useful unit for small laboratories and/or schools.

Another year of testing the antifertility drug ENOVID on five strains of mice has failed to give any evidence of carcinogenicity in doses up to the level that prevents breeding. By giving the drug in NCI pellets we have not confirmed Dunn's findings of cervical changes observed when the drug was fed in Metracal.

1. Phyllis B. Blair, David W. Weiss, and Gilbert H. Smith: Studies on antigenic cross-reactivity of oncogenic RNA viruses. Israel J of Med Sci 6: 611-616, 1970

2. V. J. Evans, F. M. Price, H. A. Kerr, and H. Montes de Oca: Effect of 7,12-dimethylbenz(a)anthracene on non-neoplastic and neoplastic rodent cells in culture. J Nat Cancer Inst 45: 429-441, 1970

3. R. R. Gantt: In vitro tRNA methylation of paired neoplastic and non-neoplastic cell cultures. Cancer Res 31: (In Press)

4. C. Hanley, and H. Hoffman: Investigations of non-human primate hemoglobin: fetal hemoglobin. Proc 2nd Conf on Exp Med Surgery in Primates (In Press)

5. W. E. Heston and G. Vlahakis: Mammary tumors, plaques, and hyperplastic alveolar nodules in various combinations of mouse inbred strains and the different lines of the mammary tumor virus. Int J Cancer 7: 141-148, 1971

6. H. A. Hoffman: Starch-gel electrophoresis of murine uninary protein. Proc Soc Exp Biol Med 135: 81-83, 1970

7. H. Hoffman and M. Rechcigl: Genetic basis of erythrocyte catalase activity in inbred mice. Enym biol clin (In Press)

9. J. L. Jackson, K. K. Sanford, and T. B. Dunn: Neoplastic conversion and chromosomal characteristics of rat embryo cells in vitro. J Nat Cancer Inst 45: 11-23, 1970

10. R. W. Pumper, P. Fagan, and W. G. Taylor: Trypsin sensitivity of mammalian cells grown in a serum-free medium. In Vitro 6: 266-268, 1971

11. C. E. Quinn, R. R. Gantt, and V. J. Evans: Comparison of methyl-labeled tRNA in a neoplastic cell line with a paired nonneoplastic control. Exp & Mol Path 13: 231-241, 1970

12. L. P. Rutzky, W. G. Taylor, and R. W. Pumper: In vitro stimulation of rat liver cells by homologous partial hepatectomy serum. In Vitro 7: 000-000, 1971

13. K. K. Sanford, B. E. Barker, R. Parshad, B. B. Westfall, M. W. Woods, J. L. Jackson, D. R. King, and E. V. Peppers: Neoplastic conversion in vitro of mouse cells: Cytologic, chromosomal, enzymatic, glycolytic, and growth properties. J Nat Cancer Inst 45: 1071-1096, 1970

14. Gilbert H. Smith, Howard B. Andervont, and Thelma B. Dunn: Attempts to detect nodule-inducing virus in strain RIII mice. J Nat Cancer Inst 44: 657-671, 1970

15. K. Stromberg: A new surfactant for isolation of the core of avian myeloblastosis virus (AMV). Fed Proc Abstracts, May-June 1971

16. W. G. Taylor: Isolation of a growth substance for mammalian cells cultures in vitro. Dissertation Abstracts, June 1971 (In Press)

17. W. G. Taylor, M. J. Taylor, and R. W. Pumper: Separation and preliminary analysis of a growth substance from Bacto-peptone. Abstract #125, 22nd Meeting of Tissue Culture Association, June 1971

18. G. Vlahakis and W. E. Heston: Spontaneous cholangiomas in strain C3H-A^{vy}fB mice and in their hybrids. J Nat Cancer Inst 46, 677-683, 1971

19. G. Vlahakis, W. E. Heston, and G. H. Smith: Strain C3H-A^{vy}fB mice: Ninety percent incidence of mammary tumors transmitted by either parent. Science 170: 185-187, October 1970

20. Nelson A. Wivel and Gilbert H. Smith: Distribution of intracisternal A-particles in a variety of normal and neoplastic mouse tissues. Int J Cancer 7: 167-175, 1971

Serial No. NCI-3208
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The study of the inheritance of mammalian proteins and their relationship to the incidence of tumors

Principal Investigators: Harold A. Hoffman and Marcia Litwack

Other Investigators: Wendel Niemann (New York University Medical School) with collaborative research with Gilbert H. Smith, Kurt J. Stromberg, and Carey Hanley (University of Illinois Medical School)

Man Years:

Total:	5
Professional:	2
Other:	3

Project Description:

Objectives: To study by methods appropriate to biochemical and quantitative genetics the inheritance of proteins in the mouse and other mammals, and to determine the genetic and non-genetic relationships between the proteins and tumors. To determine the primary structure of the normal protein and aberrant or mutant protein in order to define the gene or genes controling their production and determine the time of gene action during the development of the animal.

Methods: In these investigations mice from 18 inbred strains and substrains, which represent at least 6 different lines of known origin, are used as sources of proteins and for the production of isologous antisera. New Zealand white rabbits of known immunoglobulin type are used for the production of heterologous antisera, and non-human primates of the order anthropoid used as a source of protein.

Immunochemical methods, agar-gel-double diffusion and immunoelectrophoresis, are used in the detection and characterization of different proteins. Physical and chemical techniques of starch-gel electrophoresis, agar-gel-electrophoresis, block electrophoresis, and chromatography, both gel filtration and ion exchange, are used to isolate and classify different proteins. The fine structure and minute differences between closely related proteins are determined by peptide map analysis (fingerprints) and amino acid analysis.

Inheritance of murine urinary protein: The components of the urinary protein complex for both the 1a and 1b genetic types have been isolated by

ion exchange chromatography. The carbohydrate composition of the mouse urinary protein has been determined and only one component of the complex has been found to be a glycoprotein. Preliminary observations have shown differences in peptide mapping between the components from the two genetic types. Further studies involving peptide mapping and amino acid composition are in progress. Also efforts are being made to determine whether or not immunological differences exist between the components of the two genetic types.

Inheritance of catalase in inbred mice: Linkage studies between the low liver catalase gene, Ce, and pallid, pa, and lethal yellow, A^{y}, both on linkage group V, failed to establish linkage of Ce with these markers. This observation means that the Ce gene and the radiation induced acatalasemia mutant, C^{D}_{s}, are either on different chromosomes or are greater than 50 mup units apart. F_2 generation data from BALB/c x C57BL/6, BALB/c x C57BL, and C57BL/6 x C57BL indicate that the Ce locus may contain a multiple allelic series with at least 3 alleles regulating the turnover of the enzyme. Preliminary results on enzyme activity of hepatomas in the C3H-A^{vy}/He high incidence strain are that the hepatoma #12747 has about 25% of the enzyme activity of the liver of the host animal.

Computer techniques and data acquisition: Considerable time has been devoted to the design and analysis of experimental methods for protein determination, nucleic acid determination, and enzyme kinetics.

A new computer program has been written for the analysis of first-order reaction rates for catalase. With the computer system, we are able to complete in one day the number of assays which would ordinarily require three days.

Studies of 4S RNA in RNA tumor viruses:

The object of this study is to determine the role of 4S RNA, which has been found in all RNA tumor viruses in the viral life cycle. Avian Myeloblastosis Virus is being used as a model system. This project was initiated in the past couple of months, therefore only very basic experiments with the Avian Myeloblastosis Virus in collaboration with Dr. Kurt Stromberg are under way. The first step in the program is a comparison of the RNA profile from the intact virion and from viral cores isolated by a method developed by Dr. Stromberg.

Proposed Course of Project:

Carbohydrate composition peptide map analysis, and production of antibody specific to each isolated component of the murine urinary protein complex will continue. In vitro analysis of the genetic regulation of catalase synthesis and turnover in HTC rat hepatoma cells will be initiated this summer. In addition, attempts will be made to start primary cell cultures of fetal and neonatal mouse liver and kidney in order that these studies can be repeated with additional genetic markers. Future work on 4S RNA in RNA tumor viruses include a comparison between host cell (myeloblast) 4S RNA and viral 4S RNA

and hybridization studies using viral 65S RNA in order to determine whether or not the 4S RNA found in RNA tumor viruses are of host origin or of viral origin. The hybridization studies will involve setting up a system for *in vitro* culture of myeloblasts. This project ties in with the general objectives of this section by serving as a model for studies using similar techniques in mammalian cell culture.

Publications:

Hoffman, H. A.: Starch-gel electrophoresis of murine urinary protein. Proc Soc Exp Biol Med 135: 81-83, 1970

Hanley, C. and Hoffman, H.: Investigations of non-human primate hemoglobin: Fetal hemoglobin. Proc 2nd Conference on Exp Med Surgery in Primates (In Press)

Hoffman, H. and Rechcigl, M.: Genetic basis of erythrocyte catalase activity in inbred mice. Enym biol clin (In Press)

Serial No. NCI-3251
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Testing for Possible Carcinogenic Action of Enovid in Mice

Principal Investigator: W. E. Heston

Other Investigators: G. Vlahakis

Project Description:

Objectives: The aim of this study is to ascertain whether or not the antifertility drug, Enovid, so widely used in women, has any carcinogenic effect when fed to mice.

Methods: The Enovid is fed in the NCI mouse food in three dosages:

5 µg Enovid/gm food
10 µg Enovid/gm food
20 µg Enovid/gm food

Assuming that a mouse eats 2 gm food/day, the daily doses would be 10, 20, and 40 µg Enovid/day. The lowest dose is approximately comparable on a weight basis to the dose given women.

In addition to the original four strains A, BALB/c, C3HfB, and C57BL, used in this study we have also included strain C3H primarily to test the possible effect of Enovid on occurrence of mammary tumors. Only the highest dose of Enovid is given to the C3H females.

Results: Many of the mice of this study are now approaching 24 months of age and many have been autopsied. Tumors of many kinds have been observed in these mice but thus far we have no evidence of a higher incidence of any kind in the mice receiving Enovid than in the controls. Particularly significant is the fact that while we have seen lesions of the cervix of the BALB/c females they have not been limited to those on Enovid as Dr. Dunn reported. In fact thus far all we have seen have been in the controls. It should be emphasized that Dr. Dunn gave the Enovid in Metracal to her mice, whereas we have incorporated the Enovid in our usual NCI pellets.

Significance of the Program to the Institute:

Because of the importance of oral contraceptives in attempting to control the population explosion throughout the world, it is of utmost importance that someone at NCI investigate their possible carcinogenic effects in order that more educated decisions can be made regarding their use.

Proposed Course of Experiment:

It will still be another year before we have the data to tabulate.

Serial No. NCI-3253
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The influence of age and sex on hepatocarcinogenesis induced by chemical carcinogens

Principal Investigators: Kurt Stromberg and Melvin D. Reuber

Man Years:
Total: 2
Professional: 3/4
Other: 1-1/4

Project Description:

Reports have been made of studies on the influence of age and sex in hepatocarcinogenesis in Buffalo strain rats ingesting diethyldnitrosamine (Reuber, M. D. and Lee, C. W., J Nat Cancer Inst 41, 1133-1140, 1968) and N(4-fluoro-4-biphenyl)acetamide (Stromberg, Kurt, and Reuber, M. D., J Nat Cancer Inst, May 1970). A manuscript is in preparation on the age and sex dependency of hepatic tumors induced by 3'-methyl-4-dimethylaminoazobenzene, and an experiment is in progress with dimethylnitrosamine.

Methods: Inbred Buffalo strain male and female rats (12 to 16 rats per group) of ages 4, 8, 12, 24, and 52 weeks were fed 0.0116% 3'-methyl-4-dimethylaminoazobenzene in a semi-synthetic diet for 12 weeks. Three months later the animals were killed and a complete autopsy was performed. Currently similar groups of Buffalo rats have ingested 0.0014% dimethylnitrosamine, and three months later the animals were killed and a complete autopsy was performed.

Results: Gross autopsy and light microscopic findings on rats ingesting 3'-methyl-4-dimethylaminoazobenzene indicated 1) cholangiocellular carcinomas occurred more frequently than hepatocellular carcinomas; 2) hepatic cirrhosis was often present; and 3) the incidence of hepatic tumors and cirrhosis was highest in young male rats and lowest in old female rats. Preliminary results indicate that at a dosage of 0.0014% dimethylnitrosamine possesses minimal hepatocarcinogenic potential in any of the age or sex groups. However, male animals in younger age groups infrequently had sarcomas of the stomach and intestinal tract.

Significance of the Program to the Institute:

This series of pathologic studies in the first to evaluate the fundamental biologic parameters of age and sex in a uniform and comprehensive manner with representatives of three widely different classes of chemical carcinogens.

Publications:

Stromberg, Kurt, and Reuber, Melvin D.: Influence of age and sex in hepatocarcinogenesis induced by chemical carcinogens: Ingestion of N-4-(4'-fluorobiphenyl)acetamide by Buffalo strain rats. J Nat Cancer Inst, May, 1970

Reuber, Melvin D. and Stromberg, Kurt: Influence of age and sex in hepatocarcinogenesis induced by chemical carcinogens: Ingestion of 3'-methyl-4-dimethylaminoazobenzene by Buffalo strain rats. Submitted to J Nat Cancer Institute.

Serial No. NCI-3254
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1961

Project Title: An ultrastructural, biochemical, and immunological characterization of avian myeloblastosis virus (AMV) I. Isolation of cores. II. Localization of the 4S RNA. III. Identification and localization of enzymatic capacities.

Principal Investigator: Kurt J. Stromberg

Man Years:
Total: 3
Professional: 3/4
Other: 1-3/4

Project Description:

The recent identification of a new surfactant for isolation of the core of Avian Myeloblastosis Virus and the development of optimal methods for its use has opened the way to characterization of the two major subcomponents of AMV, a representative type C particle among the RNA tumor viruses. By surfactant-mediated removal of the envelope constituent of AMV, a partial assessment of the core constituents in ultrastructural, biochemical, and immunologic terms i now possible.

Methods and Results: The methods which led to the selection of an appropriate surfactant for core isolation are described in a previous annual report (NCI-3247).

Upon removal of the envelope, ATPase activity is obliterated, RNA dependent DNA polymerase specific activity is increased 6-fold, identifying this enzyme as a core constituent, and group-specific antigenicity is maintained, establishing the core location of the Gs antigen. Phospholipid assay of the core component reveals no phospholipid content. In an effort towards further identification of enzymatic capacities of the virus and their localization with the virion structure collaboration with Dr. Raymond Gantt of this Laboratory has led to the discovery of an enzymatic capacity of tRNA methylation for AMV virions. In conjunction with Dr. Marcia Litwack, also of this Laboratory, studies are in progress on the 4S transfer RNA reported in this virus in an attempt to localize it as a core or noncore constituent. This has importance insofar as it brings knowledge to bear on the role of the 4S transfer RNA in the virus life cycle. Is there fortuitous incorporation of cellular 4S tRNA into the virus during virus assembly at the cell membrane, or in contrast does the core location of this constituent imply that it is virus-coded and consequently essential for completion of the viral-life cycle?

Signifcance of this Project to the Institute:

A rapid, simple, and quantitative method has been developed for separation of the principal components of a representative C-type RNA tumor virus. This procedure will assist characterization of what is "host-contributed" (envelope?) and what is "virus-specified" (core) in the AMV virion.

Publications:

Stromberg, K.: A new surfactant for isolation of the core of avian myeloblastosis virus (AMV). Fed Proc Abstracts, May-June'71

Stromberg, K.: Removal of the envelope of avian myeloblastosis virus (AMV) by surfactant treatment. In preparation.

Stromberg, K., Gantt, R. R., and Wilson, S. H.: Isolation and partial characterization of the core of avian myeloblastosis virus. In preparation.

Stromberg, K. and Litwack, M.: Localization of the 4S transfer RNA of avian myeloblastosis virus. In preparation.

Gantt, R. R., Stromberg, K., and Montes de Oca, F.: A specific RNA methylase associated with Avian Myeloblastosis Virus. Submitted to Nature.

Serial No. NCI-3255
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A correlative light and electron-micrographic study of hepatocarcinogenesis induced by chemicals

Principal Investigators: Kurt Stromberg (GL&C), Melvin D. Reuber and Gary Williams (Etiology Area, NCI)

Man Years:
Total: 2
Professional: 1/2
Other: 1-1/2

Project Description:

Electron microscopic studies have failed, perhaps due to lack of a model system, to reveal pathognomonic ultrastructural features which distinguish normal or hyperplastic cells from chemically-induced neoplastic cells. Two projects, one in vivo and one in vitro, were developed as model systems to look for early and specific morphologic changes in neoplasia. It is known that hyperplastic nodules induced in rats by N-2-fluoroenyldiacetamide progress to small hepatic carcinomas. The objective of this in vivo project is to evaluate this progression, which has been studied at the light micrographic level (Reuber, M. D., J Nat Cancer Inst 34, 697-723, 1965), in terms of a correlative light and electron microscopic study that uses tumor transplantation for biologic confirmation of the morphologic diagnosis. The in vitro project is based on the development by Dr. Gary Williams of a tissue culture cell line which retains characteristics of hepatocytes, particularly a polygonal cell shape and glucose-6-phosphotase activity. This permits a morphologic evaluation of a limited number of cells at periodic intervals after exposure to chemical carcinogens and before subsequent placement in recipient rats to determine the malignant potential of the cells.

Methods: In the in vivo study three-month-old male AxC inbred rats were fed 0.025% N-2-fluorenyldiacetamide in a semi-synthetic diet for 16 weeks. Between the end of the 20th week and the 50th week autopsies were performed on several rats at successive 3-week intervale. Those hepatic lesions which were one centimeter or less in diameter, and grossly appeared to be either hyperplastic nodules or small carcinomas were bisected. One-half was divided and transplanted into two recipient rats; a tiny piece of the other half was prepared for electron microscopy, while the remainder was prepared for light microscopy. The use of thick plastic sections, about one micron in thickness and stained with toluidine blue, permitted precise correlation between these two conventional forms of morphologic evaluation and overcame the problem of random selection of cells.

Results: Preliminary findings in the rat hepatoma system include 1) irregular swelling of lysosomes; 2) an increase in thickness of the plasma membranes which frequently balloon out as protrusions into the bile canaliculi; and 3) the occurrence of bizarre cytoplasmic structures, probably lipid accumulations, which are at times enveloped by concentric layers of smooth membranes near the region of the Golgi apparatus. Whether these findings represent toxic effects of the carcinogen and are associated with regression, or are a characteristic phase of malignant change will be determined by the transplantation results and more extensive morphologic evaluation.

Electron microscopic observation of the tissue culture cell line of Dr. Williams reveals the following salient ultrastructural characteristics: 1) a homogeneous population of epithelial cells in which the endoplasmic reticulum is saculated; 2) a frequent observation of cytoplasmic fibrals interdigitating with communicating cells; and 3) dense granules suggestive of glycogen formation. In view of the preservation of enzymes characteristic of hepatocytes, such as glucose-6-phosphatase, and the persistence over long-term culture condition of a diploid chromosome number of 42, these cell lines are among the first to represent the in vitro growth of epithelial cells of hepatocitic origin.

Significance of this Project to the Institute:

Technical procedures and model systems were developed to detect the existence of morphologic correlates to carcinogenesis induced by chemicals.

Publications:

Williams, Gary; Stromberg, Kurt; and Martin, John: Some Properties of Epithelial-like Cells from Rat Liver in Long-term Tissue Culture. Submitted to Laboratory Investigation.

Serial No. NCI-3256
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructure and Biology of Mammary Tumor Virus-host Cell Interactions

Principal Investigator: Gilbert H. Smith

Other Investigators: Dr. W. E. Heston, Dr. Nelson A. Wivel, Dr. Harold A. Hoffman, and Dr. Raymond Gantt.

Collaborating Units: Dr. Phyllis B. Blair, University of California, Berkeley
Dr. Edward L. Kuff, Laboratory of Biochemistry, NCI
Dr. Harvey Ozer, Laboratory of Biochemistry, NCI

Man Years:
Total: 5
Professional: 3
Other: 2

Project Description:

Objectives: To elucidate the aspects of cell-virus interaction in the mammary tumor virus infection.

Major Findings: With Dr. W. E. Heston - hybrids between C3H-A^{vy}fB mice and C3H/StWi mice and backcross progeny with the C3H/StWi parents were produced to establish the role of the A^{vy} gene in the 100% mammary tumor incidence observed in C3H-A^{vy}fB and C3H-A^{vy}fB x BALB/c hybrids. The question is put as to whether the high mammary tumor incidence segregates with the A^{vy} gene or does the C3H-A^{vy}fB subline harbor a highly potent nodule-inducing virus (NIV). Results are as yet preliminary.

In collaboration with Phyllis B. Blair and others at the University of California, Berkeley, it has been established that the viral antigens of MTV from a series of inbred mouse lines cross-react and are closely related. A comparison of this relationship with leukemia virus of various mouse lines has been published.

A study to perfect the isolation of mammary tumor virus cores (intracytoplasmic A particles) in collaboration with Dr. Nelson A. Wivel is nearing completion. These particles have been found to be remarkably stable under a variety of conditions. Immunologic comparisons of these particles and intracisternal A particles found in a variety of mouse tissue has been performed with the cooperation of Drs. Edward Kuff and Harvey Ozer in the

Laboratory of Biochemistry; the results are still preliminary but it appears that no common antigen is shared between these particles. Studies are planned to compare these two types of particles derived from the same cell type.

A study on the distribution of intracisternal A particles in normal and neoplastic mouse tissues in collaboration with Dr. Nelson A. Wivel has been completed. The particles were found in normal and neoplastic tissues derived from all three primordial germ layers. The results suggest a vertical transmission of this ubiquitous agent. However, an indication of its biological function, if any, is still lacking. A manuscript has been published on these studies.

Nucleic acid methylase activity in normal, neoplastic, and preneoplastic mouse mammary tissues and in some of the murine and avian tumorigenic viruses is under investigation in collaboration with Dr. Raymond Gantt. No definite pattern of methylation has been established in normal vs. neoplastic tissue as yet, but a specific methylase activity has been identified in two of the avian leukoses complex viruses, Studies are in progress to extend these observations to the murine oncogenic viruses, and to elucidate the role of this enzyme in the neoplastic transformation.

Immunologic studies involving the production of enzyme-labelled antibodies for the identification and localization of antigenic sites in cells at the light and electron microscope with Drs. Harold A. Hoffman and Nelson A. Wivel have continued. Dr. Hoffman and Dr. Smith have demonstrated that peroxidase-labelled anti-MTV antibodies will specifically stain budding and mature MTV virions and that they are active in immunologic tests specific for MTV antigens. In addition a separation of conjugated γ globulins from unconjugated γ globulins has been effected. A manuscript on these preliminary studies is in progress.

Publications:

Gilbert H. Smith, Howard B. Andervont, and Thelma B. Dunn: Attempts to detect nodule-inducing virus in strain RIII mice. J Nat Cancer Inst 44: 657-671, 1970

G. Vlahakis, W. E. Heston, and G. H. Smith: Strain C3H-A^{vy}fB mice: Ninety percent incidence of mammary tumors transmitted by either parent. Science 170: 185-187, 1970

Nelson A. Wivel and Gilbert H. Smith: Distribution of intracisternal A-particles in a variety of normal and neoplastic mouse tissues. Int J Cancer 7: 167-175, 1971

Phyllis B. Blair, David W. Weiss, and Gilbert H. Smith: Studies on antigenic cross-reactivity of oncogenic RNA viruses. Israel J of Med Sci 6: 611-616, 1970

Serial No. NCI-3259
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Host genome-viral relationship in mammary tumor development in mice.

Principal Investigators: George Vlahakis and W. E. Heston

Other Investigators: Dr. Gilbert Smith

Man Years:
Total: 2
Professional: 1
Other: 1

Project Description:

Our work this year with mammary tumors in mice is going forward with several new experiments well underway. Our interest has been focused upon the relationship between the genetic factors and the viral influence in the development of mammary tumors in strains C3H-A^{vy}fB and C3HfB.

Methods: As an extension of the work that was published in Science (October 1970), in which we reported on a ninety percent incidence of mammary tumors occurring in strain C3H-A^{vy}fB females in the absence of the milk borne mammary tumor virus (MTV), we hope to have more information on the role of the $\underline{A^{vy}}$ gene and its relationship to the viral factor, the nodule inducing virus (NIV) that is found in the mammary tumors. The comparable C3HfB agouti strain, also without milk borne MTV, has a mammary tumor incidence of forty percent, occurring at an average tumor age of 19 months, compared with an average tumor age of 15 months for the high mammary tumor strain C3H-A^{vy}fB.

Strain C3H-A^{vy}fB was outcrossed to the agouti strain C3H/StWi (in which mammary tumors have ceased to develop, and in which no MTV or NIV has been demonstrated) to produce the F_1's that are phenotypically yellow ($\underline{A^{vy}}$) and have NIV from strain C3H-A^{vy}fB. The F_1's were then backcrossed to strain C3H/StWi to give two classes of backcross animals: viable yellow (A^{vy}A) and agouti (AA). NIV from strain C3H-A^{vy}fB will be present in both groups.

The females are allowed to have only 2 litters so as to make possible a comparison to the earlier published work. If the incidence of mammary tumors in strain C3H-A^{vy}fB is primarily a genetic effect of the $\underline{A^{vy}}$ gene, e.g., possibly through its enhancement of the usually poor oncogenic ability of NIV, then many more mammary tumors will occur in A^{vy}A females and at a lower average tumor age, than in the AA females. If on the other hand C3H-A^{vy}fB NIV represents a variant with a high tumorigenic capacity, then the occurrence of

mammary tumors should be approximately equal in both backcross groups of females.

Through immunological techniques we also plan to obtain a quantitative measure of the type B particles in both groups of backcross mammary tumors. The A^{vy} gene may act so as to increase NIV. Tumors from each backcross group will also be examined electron microscopically by Dr. Gilbert Smith for the presence of type B particles.

It is also quite possible that the A^{vy} gene may be acting in some other way to cause the high incidence of mammary tumors in strain C3H-A^{vy}fB as compared with strain C3HfB.

In another experiment in progress we hope to compare NIV from strain C3H-A^{vy}fB with NIV from strain C3HfB in genetically similar hosts. Strain BALB/c females, which have been shown not to have MTV or NIV, were foster nursed upon F_1 hybrids between strains C3H-A^{vy}fB and BALB/c, and F_1 hybrids between strains C3HfB and BALB/c. There is some evidence that NIV can be transmitted by the milk of hybrid females (which is the usual mode of transmission of MTV) to suckling animals. If this should prove successful then a comparison of A^{vy}NIV in BALB/c to C3HNIV in BALB/c is possible. This should answer the question as to whether strain C3H-A^{vy}fB harbors a more active NIV to account for the higher mammary tumor incidence compared with strain C3HfB.

The mammary tumor viruses that occur in the different mouse strains have also been of great interest to us. During the past year we have developed strain BALBcfA (with A MTV) to compare with MTV's in genetically similar hosts from strains C3H and DD.

Results: The evidence thus far suggests that the MTV from the high mammary tumor strain C3H is equal to or slightly more potent than MTV from the intermediate mammary tumor A strain. The incidences of mammary tumors in strains BALB/cfC3H and BALB/cfA are 100%, but the average tumor age for BALB/cfA is approximately 8.1 months as compared with 7.1 months for strain BALB/cfC3H. Both C3H and A MTV, however, are more potent than DD MTV where the incidence of mammary tumors in strain BALB/cfDD is approximately 100%, but the average tumor age is 9.3 months.

Strain BALB/cfC3H females (with C3H MTV) even when being kept as virgins still have approximately a 100% incidence of mammary tumors, but the tumors occur at an average tumor age of 9.0 months as compared with 7.1 months for the breeders.

Significance of Program to the Institute:

It is becoming clear that many tumors are not the result of the virus alone or the host genotype alone, but both are closely allied. Many of the oncogenic viruses are transmitted vertically as genetic factors are transmitted and have a very close relationship to certain genes or segments of the chromosome. The mammary tumor virus (MTV) in strain C3H-A^{vy}fB constitutes a

good model system for studying the host genome, in this case the A^{vy} gene, control of the virus. The problem can also be approached by comparing the MTV from different inbred mouse strains with different genotypes by transferring the MTV to a common mouse strain as we have done.

Publications:

G. Vlahakis, W. E. Heston, and G. H. Smith: Strain C3H-A^{vy}fB Mice: Ninety Percent Incidence of Mammary Tumors Transmitted by Either Parent. Science 170: 185-187, October 1970

G. Vlahakis and W. E. Heston: Spontaneous Cholangiomas in Strain C3H-A^{vy}fB Mice and In Their Hybrids. J Nat Cancer Inst 46, #3: 677-683, 1971

W. E. Heston and G. Vlahakis: Mammary Tumors, Plaques, and Hyperplastic Alveolar Nodules in Various Combinations of Mouse Inbred Strains and the Different Lines of the Mammary Tumor Virus. Int J Cancer 7: 141-148, 1971

Serial No. NCI-3260
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Mammary Tumors in Resistant Strain C57BL Mice

Principal Investigator: W. E. Heston

Other Investigators: G. Vlahakis, G. Smith

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives: The aim of this study has been to attempt to induce mammary tumors in Moore's line of strain C57BL mice (C57BL/M) with pituitary isografts, to compare the induction in this line with that in our strain C57BL/He and to see if the tumors of either line carry the mammary tumor virus.

Methods: Twenty-six C57BL/M females were used for this study. At from 2 to 3 months of age each female received under the capsule of the kidney isografts of 5 pituitaries from C57BL/M females of comparable age. The females were then set aside as virgins to see whether or not they would develop mammary tumors. For comparison 7 C57BL/He females likewise each received isografts of 5 pituitaries from other C57BL/He females of comparable age. Tumors that have arisen in the two lines have been examined with the electron microscope by Dr. Gilbert Smith to see if they contain viral particles.

Results: Mammary gland tumors did occur in the C57BL/M females with the pituitary isografts. In fact, they have occurred in approximately 50 percent of them but at an advanced age. Of the seven C57BL/He females, 2 have developed mammary tumors, Almost all of these mammary tumors have been adenoacanthomas but two in the C57BL/M females have been adenocarcinomas. In all females the transplanted pituitary glands have become adenomatous. In all the breeding C57BL/He females we have had we have observed mammary tumors in only 2 females but have already found 3 C57BL/M breeding females with mammary tumors. In none of these tumors, however, has Dr. Smith been able to find any type B viral particles.

Significance of the Program to the Institute:

Dr. Moore has contended that mammary tumors never occur in his C57BL/M mice either spontaneously or when they were bearing pituitary isografts. Yet he has used this line to step up the MTV for his biologic assays of the virus.

It has seemed impossible to us that one could have a strain so susceptible to the virus yet so resistant to the induction of mammary tumors with the pituitary isografts. The fact that we did get mammary tumors in 50 percent of the females fits into our general experience of induction of mammary tumors. The C57BL/M females are more susceptible to the induction of mammary tumors with pituitary isografts just as they are more susceptible to the virus. The fact that Dr. Smith has failed to find viral particles of type B is in line with our contention that one can induce mammary tumors in the mouse with pituitary isografts even without the virus.

Serial No. NCI-3261
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical and ultrastructure studies on cytoplasmic DNA

Principal Investigator: Gilbert H. Smith

Other Investigators: Gabrielle R. Neises, collaborative research with Dr. Walter C. Schneider and Dr. William R. Kidwell

Project Description:

Chemical and autoradiographic studies on the localization of DNA to membraneous components of the cell cytoplasm are in progress. Superovulated rat ovaries from immature animals are collected at various times after stimulation with luteotrophic hormones. Cellular ultrastructure and morphologic differentiation are compared with the appearance and quality of known microsomal enzymes. In addition, autoradiographic procedures are used to localize incorporated H^3-labelled nucleotides during these developmental stages.

Serial No. NCI-3211 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies, by tissue culture and cell culture methods and by amplifying methods, of the malignant cell and of the normal cell from which it arises; studies on the mechanism of transformation of the normal cell into the malignant or cancer cell; studies on characterization and identification of such cells; studies on associated significant subjects.

Principal Investigators: V.J. Evans, K.K. Sanford, R.R. Gantt, W.G.Taylor, F.M. Price, C.E. Quinn, and M.L. Taylor (Guest Worker).

Other Investigators: See individual project reports Nos. 3212, 3213, 3234A and 3257.

Man Years: July 1, 1970 through June 30, 1971

Total:	17-1/2
Professional:	9-1/2
Other:	8

Outline of Activities:

The long-term objectives of the personnel of the Section are indicated in the general title above and are described in detail in the individual projects. To carry out the objectives considerable time has been spent in the past years, and will continue to be spent as required in order to maintain and develop superior technology and instrumentation, to advance current problems, and to anticipate maintaining eminence in the primary interests.

Some of the inordinately time consuming but necessary details on acquiring and evaluating equipment and supplies for long-term, large-scale, antibiotic-free tissue culture work are mentioned here because they result in recently designed equipment and quality control supplies that other investigators in related fields find useful. Further, much time is devoted to supplying information to new investigators in other laboratories where tissue culture work is being established.

Moving, setting up, purchasing, standardizing, and recalibrating instrumentation equipment in the laboratory quarters has called for much investment of time.

The unique glassware washer purchased on research and development contract was installed with minimum "down time" because of pre-planning. A special distilled water tank for servicing this washer unit was specified, contracted for, and installed. The water tests and quality of glassware washing has been found to be satisfactory.

The new autoclaves of special formulation stainless steel, with special steam generators and heat exchangers for the distilled water steam for sterilizing, have been installed and after a series of minor but time consuming alterations, are working effectively. These units are servicing our Section and to a lesser degree taking care of the needs of people in other laboratories on request.

A dry heat small sterilizer of special design was specified, installed, and is working effectively.

Through the cooperation of the Instrument Shop and Glass Fabrication Unit of the Laboratory of Physiology, a facsimile of our original all glass triple distilled water unit was fabricated to fit into the limited space available. This unit has been photographed "in-house" and other investigators, for example from France, have sketched the unit for reproducing in their laboratory. Others from numerous laboratories of NIH have been supplied water from this still, particularly for use in electron microscopy work and special critical solutions. Our own success in growing cells in protein-free medium is largely dependent on the extraordinarily excellent quality of the water obtained from this glass unit. No commercial equal has ever been found.

A unique high efficiency 2-room walk-in constant temperature room with solid state controls was specified. The controls for each room are independent with appropriate back-up electrical sources. An appropriate alarm system for 24-hour monitoring is installed in the Guard Office. The room is functioning. Each room is designed to maintain a holding temperature of 37.5°C within a tolerance of ± 0.15°C, and uniformity of temperature throughout the rooms is to be held to within ± 0.25°C. To do this,6 sensor points at strategic locations with safety controllers up to 38.1°C to cut off heat entirely, were installed. The inner room is a much more accurate room unaffected by ambient temperature since the outer room serves as an air curtain or vestibule. The outer room, without any vestibule because of lack of space, therefore, is affected by ambient temperature with each entrance or exit.

After somewhat less than one year's usage, minor control problems have arisen largely suspected to arise either from 1) house current problems,or 2) lack of vestibule, or 3) total balance of electronic controls.

The rooms have been described in trade journals and are being copied for precise work in other institutes in the NIH.

Laminar flow and safety hoods and benches designed as work containment units in consultation with NIH biohazard engineers protect the investigator and cultures from most microbiological hazards. They also serve as containment boxes for bottling sterile solutions. Although effective in these respects, as well as conserving space for the operations mentioned, they are defective in several regards. This has meant considerable loss of time and much expense has been incurred to correct these. Despite precautions to the manufacturer, the boxes build up heat to 95-102°F,--a decided hazard to operators and cultures. Specification and design work to correct this

has consumed much time. This correction is not yet made. Two prototypes as designed by Environmental Services for approval are under test, to draw off heat into main plenum since air conditioning from building supply in individual cubicles is not adequate to offset "dumped" heat. Failure on the part of the company supplying hoods to counter-weight hood sashes created occupational hazard to workers from falling sash which could crush hands or arms. This one defect has been corrected in two of seven hoods with remaining five still to be corrected. Default and/or bankruptcy of original low bidding company has released them from responsibility.

A constant search at expense of time and money has been under way with us to locate fetal bovine serum capable of growing cells of many species in large and small amounts, when the serum is free of a reasonable number of viruses and testing free of mycoplasma. This is of considerable concern to all investigators. An Ad Hoc group at NIH met at our instigation to discuss ways and means of resolving this problem. Information was exchanged from personal testing of serum and general consideration of this problem. As a result a workshop is planned the end of April to delineate all the problems and to encourage cooperation from commercial sources to produce quality serum.

Sources of equipment for growing cultures both large and small under homeostatic conditions to shed light on influence on neoplastic transformation have been sought out and some integral parts are on hand particularly with regard to large masses of cells. One of these devices has potentiality not only for stabilizing culture conditions but also for growing mass amounts of cells with a lesser amount of hand labor and under more uniform conditions. The unit purchased has required some bottle cap modifications for sterilization but in general appears promising for an integral part of the total instrumentation.

The purchase order system has already been found useful, and hopefully in the near future the contract system will prove useful to reduce lead time, manpower and space in determining ways and means of controlling ready production of nonneoplastic and neoplastic clones of cells for fastidious work. Particular emphasis is being placed on determining the best medium, serum, and influence of gas mixtures including concentrations of O_2 and N_2. More details are available for accomplishments in Serial No. NCI-3213.

A CO_2 incubator has been located and installed sufficiently small for space alloted. This will help immensely in the plate cloning procedures for special carcinogenesis studies, among other uses.

A small, accurate, readily manipulable, and economical microcinematographic unit has been sought for some time. Considerable evaluation of this type unit has been made. One has been selected. It will also be equipped with a unique temperature controlled stage only recently available. It meets a real need we have had for several years to analyze in more detail dynamic morphology and growth characteristics of living non-malignant and malignant cells from the same common origin. Further, it will be useful in chemical carcinogenesis studies, migration rates, etc.

Consultation and technical advice has been given to the fabrication of our constant temperature room regarding a portable incubator designated as a Portabator. The unit is on wheels and is readily moved close to or into a sterile room. The unit is powered with a readily purchased heavy duty automobile battery,or line current can be used if available to recharge battery or used alternately in lieu of battery. The instrument is controlled to within $\pm$ 0.5^{o}C. This item has been evaluated in the laboratory. It has excellent potential for usage in small laboratories, schools, general field service, and in large laboratories where critical attention to fluctuation in temperature at fluid renewals is significant.

Serial No. NCI-3212, Project 1 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project 1, Title: Studies of cell nutrition and metabolism in vitro.

1. To test ability of cells to grow in completely chemically defined medium.

2. To study growth and metabolism of a neoplastic C3H/HeN mouse cell line with special emphasis on vitamin B12, folic acid interrelationships, and nucleic acid precursors.

3. To determine the effect of fractions of horse and fetal calf serum on growth of freshly explanted C3H mouse embryo cells.

4. To test ability of C3H mouse embryo cells to grow in medium containing gelding mare or stallion serum obtained from a commercial source.

Principal Investigators: V. J. Evans and F. M. Price

Other Investigators: H. A. Kerr, R. A. Dworkin, R. R. Gantt, K. K. Sanford.

Man Years: See Serial No. NCI-3211

Cooperating Units: Nucleic Acid Section, Laboratory of Biochemistry, NCI

Project Description

Objective:

To determine in vitro nutritional requirements of freshly isolated cells and long-term lines, both neoplastic and nonneoplastic, and to improve culture conditions of the cells so that rigidly controlled cells can be used to study the differences between nonneoplastic and neoplastic or malignant cells at the nutritional, metabolic, and morphologic levels.

Major Findings:

1. Ability of cells to grow in completely chemically defined medium.

a. Growth of primary cultures: Attempts have been made to improve the formulation of our culture medium in order to provide a medium which will support growth of primary cultures without the use of serum or protein

hydrolysate. At the same time we were trying to eliminate unnecessary ingredients. Major changes thus far have involved increasing the concentration of glucose, several amino acids (particularly L-glutamine) and the B vitamins and adding linoleic acid, linolenic acid, pyruvic acid, and putrescine. Components deleted from the medium include 6 coenzymes, 3 nucleic acid precursors, and 3 amino acids.

Results thus far have been encouraging although no established or long-term cell lines have been obtained. Our best improved medium to date has supported growth of primary C3H mouse embryo cultures for 7 culture generations over a period of approximately 8 weeks. This compares favorably with the chemically defined medium, NCTC 135, which we have been using which has supported growth of primary mouse embryo cultures for 2 or 3 culture generations before they finally died out or lived indefinitely but only with assiduous efforts.

b. Growth of established cell lines: To date only one cell line has been tested by quantitation to determine its growth in our newer formulations. The line tested was Earle's strain "L" 2071 cells which have been growing in chemically defined medium for over 15 years. Initial tests have been inconclusive and over a period of 7 days growth in medium 135 was as good but probably not significantly better than that in two media of improved formulas in which the freshly implanted cells grew well. Since the 2071 cells have been grown in medium 135 for so many years, a characteristic period of adaptation appears necessary in order to reveal improved growth in a new medium formulation. Growth, however, of the S3 line of HeLa adapted earlier to NCTC 135 alone has been more responsive to the new formulations than to medium NCTC 135. Quantitative analysis is in progress.

2. Study of growth and metabolism of a neoplastic C3H mouse cell line with special emphasis on vitamin B12 and folic acid interrelationships and nucleic acid precursors.

a. Growth studies: Growth studies this year were limited to a determination of the concentration of vitamin B12 actually required for growth under conditions requiring vitamin B12. It was found that a concentration of 0.73×10^{-6} M would satisfy this requirement. It was also found that methyl-B12 and hydroxocobalamin would also satisfy the requirement at the same concentration.

b. Metabolic studies: The amount of DNA synthesized by cells in the presence of deoxyuridine and folic acid at a concentration of 0.57×10^{-6} M was limited. It could be increased by adding vitamin B12 or substituting thymidine for deoxyuridine. When vitamin B12 was present but the folic acid concentration was lowered to 0.57×10^{-7} M, less DNA was synthesized and the decrease was partially reversed by replacing deoxyuridine with thymidine. Incorporation of radioactivity from both ^{14}C formate and deoxyuridine-2-^{14}C into DNA-thymidine was limited in the absence of vitamin B12. It appears that vitamin B12 functions, in part, to increase DNA-thymidine synthesis in these cells by raising limiting levels of folate coenzymes that are required in the metabolism of "one-carbon fragments."

3. Effect of large and small molecule protein fractions of horse and fetal bovine serum on growth of freshly explanted C3H/HeN mouse embryo cells.

It was determined over a period of more than two years that the mouse embryo cells would grow much better if the large molecule fetal bovine fraction was in the medium. Adding the small molecule fraction of either fetal bovine or horse serum did not appreciably improve the ability of this medium for growth of the cells. The large molecule fraction of horse serum alone or in combination with either the small molecule fraction of horse serum or fetal bovine serum supported limited growth of these cells until the cells ultimately adapted to these media and grew well, after a period of over one year in vitro. Cells in medium NCTC 135 with no supplement or with the small molecule fraction of either horse serum or fetal bovine serum did not grow continuously as long-term lines, although with the unsupplemented NCTC 135 no assiduous effort was made for characteristic adaptation.

4. Ability of C3H mouse embryo cells to grow in medium containing gelding, mare, or stallion horse serum obtained from a commercial source.

Three pools of C3H mouse embryo cells were set up to test stallion, mare, and gelding serums. Two of the pools were from C3H/HeN embryos and the third pool from C3H/He embryos. To date in all three pools growth in NIH gelding horse serum (control serum) is less satisfactory than in commercial gelding, mare, and stallion serum. This has resulted in an intensive survey to learn reasons for the poorer quality of NIH serum. This has been resolved and is probably one of the most important single extraneous fingings made in some time for the general use of workers in the field. Of the commercial serums in long-term experiments the best growth has been obtained with the stallion serum. The next best growth was obtained with the commercial mare serum, although the difference in growth potential between it and gelding serum was practically non-significant. The growth of all the cultures in all four horse serums is poorer than in fetal bovine serum. In stallion serum, irrespective of the size of initial population, all cells have had approximately the same rate of proliferation. The subculture interval is longer in the C3Hf/He cells than in the two pools of C3Hf/HeN in the mare and gelding commercial serums. In these two serums the size of initial inoculum seems to have an influence; the smallest inoculum appeared to grow more slowly.

Significance and Proposed Course:

1. The original major objective for which cells in chemically defined medium was initiated has not to date been completely obtained. This was to obtain nonneoplastic cell lines where nutrition was reproducible and characterized for 1) subsequent controlled treatment with carcinogens, or 2) to obtain mass amounts of tissue early in vitro to follow changes to the malignant state. Until cell lines free of type C particles can be established with no initial lag in growth and no need for characteristic adaptation, the objectives are delayed. We appear to have made a definite improvement in the formulation of our chemically defined medium, but no formulation either in this laboratory or elsewhere is yet satisfactory for

rapid growth of fresh tissue or for cloning cells in serum-free medium. Studies using the serum fractions have indicated that the components in serum of major importance contributing to growth of mouse embryo cells are probably in the large molecule fraction. Further analysis of the large molecule serum fractions may yield defined metabolites which can be used in completely defined medium. Several approaches to practical analysis are under consideration. The study involving the mare, gelding, stallion horse serum and the fetal bovine serum also indicates that there may be a hormone-like influence provided by serum which may influence the growth of mouse embryo cells. Hopefully, hormone-like or other growth stimulants, perhaps polypeptides, can be isolated from serum and used in a cleaner form as part of a completely chemically defined medium. Further, it is significant that cells grow better in fetal bovine serum which has a lower protein content than horse serum. Also significant is the fact that the mare serum was from a non-pregnant mare.

Only with a completely defined medium system free of serum is it going to be possible to rule out influence of viruses and mycoplasma on the growth of cells and any relation these may have on the propensity of cells in vitro to undergo spontaneous malignant transformation. Autoclavable peptones (see Series No. NCI-3257) have been evaluated to see if they will substitute for serum and assure removal of these agents. One other particular problem through the years has been that defined medium, in fact all media, has used a bicarbonate buffer system. Along with the studies on cell nutrition has been considered the effect of the complete or partial substitution of organic buffers for sodium bicarbonate on growth and possibly even more ready establishment of fresh tissue lines. This is significant because cells undergoing neoplastic transformation have uniformly been in bicarbonate buffered solutions. Further exploratory information on nutrition of cells and organic buffers may be seen in Series No. NCI-3257. In addition, the influence of a variety of gas mixtures has been evaluated on the efficiency of plate cloning of cells, a highly critical and definitive test for growth potential of mediums and physical conditions. The continuing program on nutrition of cells is dependent on both an analytical approach to determine growth promoting additives as well as the to date still successful synthetic approach of logical additions to formulations.

Serial No. NCI-3212, Project 2 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project 2, Title: Cell transformations in vitro.

Principal Investigators: V. J. Evans and F. M. Price

Other Investigators: R. R. Gantt, H. A. Kerr, R. A. Dworkin, and K. K. Sanford.

Cooperating Units: H. Montes de Oca, American Foundation for Biological Research, Rockville, Md.

A. Richter, Laboratory Cell Suppliers, Frederick, Md.

J. W. Hartley, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.

Man Years: See Serial No. NCI-3211.

Project Description

Objectives:

1. To study malignant transformation in vitro with:

   a. Cells initiated in chemically defined medium with no serum supplement.

   b. Cells cultured in fractions from horse and fetal calf serum.

   c. Cells exposed to action of 7,12 dimethylbenz(a)anthracene, a known carcinogenic hydrocarbon in vivo.

   d. Cells initiated in stallion, mare, and gelding horse serum.

   e. Syrian hamster cells exposed to benzpyrene.

   f. Density gradient for separating homogeneous populations of neoplastic and nonneoplastic cells from a heterogeneous population.

2. To study the response in vitro of neoplastic and nonneoplastic cells of three rodent species to small doses of DMBA as an index of neoplasia.

3. To try to distinguish between the influence of cell selection and induction on the development of malignancy in vitro.

Methods Employed:

Nonneoplastic cells in vitro were subjected to media with a variety of serum, serum fraction supplements, and without any large molecular supplement to determine the influence of these additions on these cells' ability to produce tumors. Also nonneoplastic cells were subjected to a chemical carcinogen to determine if neoplastic transformation could be induced in vitro. Experiments testing the action of dimethylbenz(a)anthracene were made with neoplastic, nonneoplastic, and freshly explanted cells to test potentiality of an in vitro test for malignancy in correlation with in vivo tests. Experiments using ficoll as a discontinuous gradient were made on paired nonneoplastic and neoplastic cell lines to determine heterogeneity of populations and to determine neoplastic potential of each population and other characteristics of isolated populations.

Major Findings:

1a. Cells initiated in chemically defined medium with no serum supplement.

To date we have had four cell strains of C3H mouse embryo cells which have produced tumors after 169 days in vitro. The other lines produced tumors from 400 to 900 days in vitro. All of the lines proved to be carrying type C virus particles and were highly aneuploid although we could find no correlation between chromosome abnormalities and virus infection with tumor production. More recently a cell line was injected at 117 days in vitro with no production of tumors. This cell line never became established and no further injections were made. Presently several more lines have been set up in completely chemically defined mediums and, hopefully, more information on neoplastic transformation in protein-free medium can be learned from these lines tested free of type C particles.

1b. Cells cultured from fractions from horse and fetal calf serum.

Twelve cell lines from a minced mouse embryo pool were maintained in chemically defined medium to which large and small molecule components (physically fractionated) of horse and fetal bovine serum were added in all combinations to determine if any fraction predisposes to neoplastic conversion or can cause repression of transformation. Tumors were obtained from four cell lines. The earliest tumor was obtained at 222 days in vitro by cells grown in medium plus both the large and small molecule fraction of horse serum. At 428 days in vitro cells grown in medium containing whole horse serum produced tumors. Cells grown in medium containing small molecule fraction of horse serum and large molecule fraction of fetal bovine serum produced tumors at 603 days in vitro. The last line to produce tumors was that grown in medium containing the large molecule horse serum fraction and the small molecule fetal bovine serum fraction. The tumors from this line were produced at 784 days in vitro. The small molecule fraction of horse serum, therefore, appears to contain the factor in horse serum that accelerates malignant transformation of cells in vitro and the small molecule fraction of fetal bovine serum may also contain this factor in lesser amounts or with lesser activity accounting for the delay.

1c. Cells exposed to action of 7,12 dimethylbenz(a)anthracene (DMBA), a known carcinogenic hydrocarbon in vivo.

A long-term, stable, nonneoplastic C3H embryonic mouse cell line in fetal bovine serum was treated with DMBA at a concentration of 1 microgram per ml of medium in 3 intermittent exposures over a period of approximately one month.

The chromosomal pattern was altered radically compared with unexposed cells of the line. The cells were highly sensitive to the chemical carcinogen with destruction of at least 90% of the population exposed. The cells obviously were transformed, and after a latent period of a year in the mouse tumors appeared in mice which had received cells 11 months after they had been treated with carcinogen.

Approximately one year (352 days) after initial treatment, the treated cells were again exposed to DMBA and to the solubilizing agent, DMSO. All animals injected with these treated cells obtained tumors. By this time, the control, untreated cells, were neoplastic and were producing tumors in host mice. The action of a single exposure to DMBA appeared to delay tumor production, while with a second dose of carcinogen the cells produced tumors with less delay of culture time and latent period.

Apparently in this instance the first treatment destroyed most of the nonneoplastic cells and after a long delayed recovery period some cells of the population were affected by the DMBA in a manner suggesting a repressor-like action of the DMBA so that they were slower to produce tumors than the control and twice treated cells. The reason for this is still unexplained.

1d. Cells initiated in stallion, mare, and gelding horse serum.

Stallion, non-pregnant mare, and gelding horse serum (Flow Laboratories), with NIH gelding horse serum and fetal bovine serum (Flow) as controls, are being tested on three independently established C3H mouse cell lines to ascertain whether differences in hormonal content, for example, have any influence on neoplastic transformation with respect ot time or degree.

To date tumors have been obtained in two of the lines established in medium containing stallion serum. These tumors were produced from cells at 158 days in vitro. One cell line in medium containing gelding horse serum produced tumors at 200 days in vitro. No tumors have been produced by cells in medium containing either mare serum or fetal bovine serum after over 400 days in vitro. Cells in medium containing NIH gelding serum have not grown well and only one line has been injected, at 200 and 275 days in vitro. No tumors have been obtained from these injections to date, although the latent period of the cells in the animals may be quite long. The current conclusion is that cells in mare serum do not become neoplastically transformed, or if so, at a time much after stallion and gelding horse serum. Prediction of malignant state of the cells by cytological analysis (see serial No. NCI-3213) is highly correlated with tumor production.

1e. Syrian hamster cells exposed to benzpyrene.

In efforts to reproduce for our particular program the work reported by others that chemical carcinogens induce malignant transformation, Syrian hamster cells and Syrian hamsters infected with Graffi agent have eeen exposed to BP under the exact conditions described by previous investigators. The results to date are described in Serial No. NCI-3213, project 1.

1f. Density gradient for separating homogeneous populations of neoplastic and nonneoplastic cells from a hetherogeneous population.

On the assumption that a heterogeneous population may contain both non-neoplastic and neoplastic cells in different ratios, efforts were made to separate populations by use of density gradients made of ficoll as described last year, grow populations in vitro, and determine if all constituent populations have the same neoplastic or nonneoplastic potential as the parent heterogeneous populations. Many technical problems developed; some have been resolved. Sterility problems with the ficoll gradient have been resolved. Single cell populations were obtained without sieving which had previously damaged cells for growth. The use of Ca+Mg-free saline aided in obtaining clump-free populations. Clumped populations had tended to slip into the next higher density layer of ficoll. Improved methods of layering ficoll with no mixing was achieved with a stabilized syringe fitted with a capillary pipette for slow and gentle layering of 15 to 30% by weight of ficoll of 6 equal concentrations and loading top of ficoll gradient with 1 to 1.5 millions; populations greater, were not readily supported and slipped through gradient. With improved methods one strain of C3H mouse cells judged by injection to be neoplastic was separated into 2 populations. One population when grown to give adequate injectable populations gave tumors; the other did not produce tumors. Cytologic examination of these populations by the method of Sanford and Barker confirmed the subsequently obtained injection results. These data are to be confirmed on synchronized cultures for cleaner separations and applied to other neoplastic heterogeneous strains and specifically to Wi-38, a line of human cells said to be nonneoplastic, with 5 to 10% heteroploid cells and 90 to 95% diploid cells. This line has a history of finite life. It will be determined if the 2 populations can be separated, if infinite life is possible with either population, and if either population in adequate minimum populations in pretreated animals will produce tumors. The potential significance is to assure that populations on injection are nonneoplastic or whether on injection the ratios of non-malignant cells to malignant cells can give an erroneous answer regarding tumorigenicity of a strain of cells. With human lines determination of the answer is difficult because pretreated animals for injection are required and only if tumors are produced can separation be assumed to have occurred. With cells from inbred animals injection data have more significance if negative injection results are obtained. Concomitant with perfection of cell population separation by density gradients will be, under purchase order system, efforts to obtain 2 populations from the Wi-38 by plate cloning despite the fact that cloning of nonneoplastic cells is difficult. An ancillary benefit from such information on Wi-38 would be better control of vaccine production, which is already being done with the mixed populations of Wi-38.

2. To study the response in vitro of neoplastic and nonneoplastic cells of three rodent species to small doses of DMBA as an index of neoplasia.

In a study using long-term mouse, rat, and hamster cell lines, toxicity of cells from exposure to 7,12 dimethylbenz(a)anthracene (DMBA) appeared to be useful as a reliable rapid assay to determine tentatively the nonneoplastic state of cell lines examined. In our system all long-term nonneoplastic cells were resistant. No cytotoxicity was initially found in freshly explanted mouse embryo cells in horse serum or fetal bovine serum systems when surely the cells were not yet neoplastic. As yet no rationale for the behavior of the short-term cultures can be offered. A manuscript has been published.

3. Efforts to distinguish between cell selection and induction as they influence development of malignancy.

Unequivocal answers to this question still await a single cell line developed in a medium and conditions that will not permit so-called "spontaneous" malignant transformation in vitro. The purchase order system is leading to improvements in cloning from populations prior to the time they undergo transformation and cloning under increasingly greater efficiency. Further, the recent findings with types of serum should keep these clones nonneoplastic. Of course, constant monitoring by the 3 methods discussed earlier will be done to determine possible malignant transformation. Multiple cloned lines in mare's serum, fetal bovine serum, and large molecule fetal bovine serum should be tested for response to stallion horse serum for its possible inductive transformation action in order to differentiate between cell selection action which may be the reasons for the respective behavior of mixed populations in these 3 serums and/or fractions. This would be a particularly attractive approach to an answer to this selection-induction question since studies with carcinogenic hydrocarbons have not been productive to date.

Significance and Proposed Course:

Continuing efforts to initiate and establish virus-free cell lines in serum-free and preferably protein-free medium are enormously time consuming and difficult to achieve. Although some lines have been initiated and established in protein-free medium, they have been found to contain type C particles and have produced tumors. Previously reported data lend no confirmation that the spontaneous transformation of cells in vitro could be the result of presence of virus. Until lines can be initiated and established free of virus in controlled medium, the answer remains controversial. One very significant finding is that lines grown in small molecule fractions of horse serum become transformed. The molecular weight of the serum fraction is below that of viruses and it appears therefore that it is not a virus responsible for the malignant transformation in this situation. Hormonal or hormonal-like action might be more reasonable or some other component in the small molecule horse serum fraction. Hence, it becomes important to analyze or fractionate further this portion of the serum as is projected. The data on the malignant conversion of cells in horse serum from males and not from females suggests some significant difference here, perhaps hormonal.

For clear-cut answers we need to establish lines in preferably protein-free or serum-free medium. With the difficulties in formulating mediums and establishing lines in protein-free medium, it becomes attractive concomitantly to try to establish lines in serum substitutes such as peptones or peptone fractions. These can be sterilized by autoclaving, removing the potential virus influence unless introduced in the original tissue. With serum autoclaving is impossible, and with serum myriads of other problems occur. The possible use of peptones or fractions thereof raises other problems because it becomes necessary and time consuming to ascertain whether cells undergo malignant transformation in them. Even if they should, it appears conceivable that analysis of the active factor, and its deletion, might be less difficult than seeking and deleting the factor in serum. Moreover, the same methodology might be used to isolate the active accelerating neoplastic factor in the serum fraction or reveal the factor in chemically defined medium.

The information that no tumors are obtained in cells in medium containing large molecule horse or fetal bovine serum, besides offering an approach to determining why this component has a repressive-like action, is technically important for all investigators employing cell culture. Vaccine production from long-term lines might be feasible. Further, it opens up avenues for giving culture sources that are not neoplastic for controlled testing experiments. If another possibility exists that shifts in pH or O_2, CO_2, and N_2 ratios are important, these too are amenable to test and some efforts are concomitantly being made to explore these (see Serial No. NCI-3213). These are under consideration not only for direct action on growth and homeostasis of cells but also for influence in spontaneous neoplastic transformation. Further, the availability of cells not transforming in mare serum and large molecule fraction of fetal bovine serum and horse serum gives us lines that can be used to study the importance of selection versus induction as the reason for malignant transformation in vitro.

The correlation between relative resistance to DMBA and the neoplastic state of long-term rodent cells appears to be a time-saving tool in neoplastic transformation studies, particularly since a further correlation can be made with cytological differences between neoplastic and nonneoplastic cells as described by Sanford and Barker (NCI-3213).

We have not been able to demonstrate neoplastic transformation by use of DMBA although chromosome studies on treated cells indicate that treated cells are altered. The cause and nature of this alteration is yet to be determined. It may be that certain critical factors such as osmolarity or pH of nutrient medium are necessary for the cells to be transformed by the carcinogen. Another possibility is that some interaction between viruses and carcinogens (see NCI-3213) brings about neoplastic transformation. Or it may be that the age of the cells in vitro at the time of treatment is critical.

Honors, Awards, and Other Activities (Dr. V. J. Evans)

1. Vice President, Tissue Culture Association, 1968-1970.

2. Vice President, Tissue Culture Association, reelected 1970-1972.

3. Member, Board of Governors, W. Alton Jones Cell Science Center, Lake Placid, New York, 1970-1972.

4. Chairman, Executive Committee, Board of Governors, W. Alton Jones Cell Science Center, Lake Placid, New York, 1970-1972.

5. Member, Committee to select additional members, Board of Governors, W. Alton Jones Cell Science Center, 1970-1972.

6. Chairman, Insurance Committee, W. Alton Jones Cell Science Center, 1970-1972.

7. Member, Endowment and Funding Committee, W. Alton Jones Cell Science Center, 1970-1972.

8. Organizer, Ad Hoc Committee on Serum Problems in Tissue Culture, February, 1971.

9. Chairman, Local Committee, Twenty-first Annual Meeting, Tissue Culture Association, Washington, D. C., 1970.

10. Consultant for Index of Tissue Culture from 1966-1969.

11. Consultant, Interagency Board, U. S. Civil Service Commission for Biology.

12. Member, Editorial Board, Journal of Cryobiology, 1969-1972.

13. Co-Organizer, Workshop on Serum Problems in Tissue Culture, sponsored by Tissue Culture Association, Division of Biologics Standards NIH, and National Cancer Institute NIH, at W. Alton Jones Cell Science Center, April 1971.

Publications:

1. Evans, V.J., Price, F.M., Kerr, H.A., and Montes de Oca, H.: Effect of 7,12-dimethylbenz(a)anthracene on non-neoplastic and neoplastic rodent cells in culture. J Nat Cancer Inst 45: 429-441, 1970.

2. Quinn, C.E., Gantt, R.R., and Evans, V.J.: Comparison of methyl-labeled tRNA in a neoplastic cell line with a paired non-neoplastic control. Exp & Mol Path 13: 231-241, 1970.

3. Price, F.M., Gantt, R.R., and Evans, V.J.: Effect of fractions of horse and fetal bovine serum on neoplastic conversion of C3H mouse cells in tissue culture. In Vitro 6: 000-000, 1971.

Serial No. NCI-3213, Project 1 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project 1, Title: Carcinogenesis in vitro.

Principal Investigator: K. K. Sanford

Other Investigators: S.L. Handleman, R.R. Gantt, V.J. Evans, M.L.Hursey, W.G. Taylor, and C.U.Garrison.

Cooperating Units: A. Richter, Laboratory Cell Suppliers, Frederick, Md.
H. Montes de Oca, Biomedical Research Institute, Rockville, Md.

Project Description

Objectives:

1. To elucidate the cause(s) of "spontaneous" neoplastic transformation of mammalian tissue cells in vitro.

2. To isolate and identify the factors in serum that influence neoplastic transformation.

3. To develop effective methods for inducing neoplastic transformation that will be both rapid and reproducible for both long-term nonneoplastic cell lines and short-term cultures.

4. To obtain clones of relatively stable nonneoplastic cells that can be transformed by methods developed in #3 and thus provide quantities of neoplastic and nonneoplastic cells from one cell for biochemical studies. The use of clones developed from one cell is essential for controlled biologic material and for eliminating the possibility that the carcinogen or carcinogenic agent is selecting certain cell types rather than inducing a neoplastic change.

Methods Employed:

Experiments were designed to examine the influence of serum as supplement to chemically defined medium on neoplastic transformation of cells in vitro and to obtain information concerning the specific serum factors that appear to accelerate or suppress "spontaneous" neoplastic transformation. In addition, chemical carcinogens were assayed on cells in primary or secondary culture to look for morphologic changes. Cells have been initiated and grown in several types of chemically defined medium without serum or other undefined supplement for assaying certain serum polypeptides. Cells taken

directly from in vivo have been plated under various conditions in efforts to establish clones of nonneoplastic cells. All tests for neoplastic transformation were made by implanting cells into syngeneic hosts.

1. Effects of serum on neoplastic transformation of cells in vitro.

The type of serum used to supplement chemically defined medium NCTC 135 (CDM) was shown previously to influence the time of neoplastic transformation of certain cells in vitro. In horse serum (HS) supplement C3H mouse embryo cells regularly underwent neoplastic transformation between 98 and 188 days in vitro, whereas cells from the same embryo cell pool grown in fetal bovine serum (FBS) in most cases either failed to transform during the test period or the transformation was greatly delayed. In two subsequent studies, calf serum (CS) from less than 6-month old animals had an effect on neoplastic transformation similar to that of HS. These observations raised certain questions which we have attempted to answer in the following experimental studies. Although some of these studies were carried out earlier, the results of animal assays were completely analyzed and cytologic studies made during the past year.

a. Reproducibility of serum effect on neoplastic transformation.

The first question was whether the serum effect was limited to embryo cells from the C3H strain of mouse or whether embryo cells from other strains of mice would show a similar response. Cell lines from embryos of 4 inbred strains, A/HeN, I/An, and germ-free ALB-M2 and C57BL/KaLwN were initiated in culture. Paired cell lines each originating from the same embryo cell mince were grown in CDM + 10% HS or FBS. Cells were assayed at intervals in syngeneic hosts. Samples of the cell suspensions used for animal assay were also prepared for cytologic examination. These preparations were coded and diagnosed for neoplastic state by cytologic criteria as part of our continued effort to correlate results of cytologic diagnoses with results of in vivo assay.

A serum effect on neoplastic transformation was clearly shown on cell lines from strain A and ALB-M2 mice, but not on cell lines from the strain I mouse, although the number of sublines was limited. Data from the C57BL cells were suggestive but too limited to be definitive. Of the 68 cytologic diagnoses, 79% were consistent with results of in vivo assay. Of the 14 inconsistent diagnoses, 7 were considered (+) and no tumor developed. However, cells of each of these lines at the subsequent injection grew as tumors. Six were considered borderline or (+) because some cells showed neoplastic characteristics. These did not produce tumors. One was diagnosed (-) and 1 of 2 animals developed a tumor after a prolonged latent period.

b. Carcinogenic activity of horse serum.

A second question was whether some component(s) of HS is carcinogenic. If so, it might be possible to demonstrate a dose response or an acceleration and/or increase in number of neoplastic transformations with increasing levels of HS. An experiment was designed to test this possibility.

A pool of cells was prepared from one C3Hf/HeN embryo. Equal volumes of cell suspension were then transferred to CDM with 10%, 20%, or 40% HS. Cells were assayed for neoplastic transformation as described in the first study. In addition, some cells were grown on 10% FBS and on 10% HS with 0.2% fetuin fraction isolated from the FBS. Since FBS differs from HS in containing fetuin as a major protein fraction, we tested whether fetuin added to 10% HS would delay or prevent the neoplastic transformation. Although there was some variation between sublines, an increase in HS concentration from 10% to 20% or 40% did increase the percentage of tumor-producing cultures and tended to shorten the latent periods for tumor development. Of all mice implanted with cells after 150 days of growth in vitro, 11% developed tumors from the two sublines on 10% HS; 68% from cells on 20% HS; and 62% from cells on 40% HS. Cells on FBS did not transform during the test period. Fetuin fraction added to 10% HS did not suppress neoplastic transformation. Whether this negative effect of fetuin was the result of the processing of the fraction (i.e., the two ammonium sulfate precipitations and dialysis) was not tested by reconstitution of fractions. Of the 58 cytologic diagnoses, 93% were consistent with results of in vivo assays. The results of this study seemed to indicate an effect of HS concentration on neoplastic transformation and thus suggested that some component(s) of HS might be carcinogenic.

c. Suppression of transformation by fetal bovine serum.

A third question was whether some component(s) of FBS suppresses neoplastic transformation. To evaluate this possibility, it would be desirable to know whether neoplastic transformation occurs regularly and early in CDM without serum supplement. The absence of serum in previous studies has not prevented neoplastic transformation; but the cells grew very slowly, transformations were manifest after many months, and at least some of the lines were found to harbor murine leukemia virus. If FBS should contain a suppressor, it might also suppress neoplastic transformation in a medium containing HS or CS. Two experiments were designed to test this possibility.

In the first, a cell line from C3Hf mouse embryos was initiated in CDM + 10% FBS. After 55 days cells tested (-) for complement-fixing mouse leukemia virus and recoverable virus. Two weeks later, cells from one culture were used to inoculate replicate cultures to be carried on the several experimental media. Cells were assayed at intervals in syngeneic hosts for neoplastic potential. Cells of both sublines on 5% HS had undergone neoplastic transformation when first assayed at 203 days in culture. Tumor latent periods were short (avg. 22 days). Cells on FBS produced no tumors. Cells of each subline on HS + FBS grew as tumors only after 301 days in culture and 16 negative injections. The 4 tumors that finally developed had long latent periods averaging 210 days. These results suggested that FBS added to HS may delay neoplastic transformation as compared with cells on HS alone.

Both sublines on 10% CS underwent neoplastic transformation by 175 and 203 days in vitro. Tumor latent periods were short (avg. 24 days). The subline on 5% FBS did not produce tumors. Both sublines on CS + FBS did not produce tumors. These results also suggested that FBS added to CS

may prevent or delay neoplastic transformation as compared with cells on CS alone. It appears that some component of FBS suppresses neoplastic transformation either directly or indirectly by interacting with some carcinogenic component(s) in HS or CS.

d. Effect of fetuin fraction.

The fetuin fraction prepared from FBS by one precipitation when added to CS appeared to suppress neoplastic transformation. In one subline no tumors developed. In the other, two tumors did arise early (latent period 55 days), but none developed from later injections.

e. Effect of prolonged culture in fetal bovine serum.

In the second experiment, we used an established nonneoplastic line of C3H mouse embryo cells that had been initiated and carried in CDM + 10% FBS for 490 days (69 subculturings) before transfer to the experimental media. Cells of this line in a previous study had been transferred to HS after 15, 60, and 90 days in vitro. All three transferred lines underwent neoplastic transformation by 113-165 days in the HS medium, a period similar to that required for transformation of cells of the same pool initiated in HS medium. After 435 days in culture, cells transferred to HS underwent neoplastic transformation by 279 days to produce a tumor with a long latent period of 100 days. In the present experiment, cells transferred to the experimental media with two exceptions did not transform. These results seem to indicate that cells maintained for long periods in FBS may be so stabilized they are less subject to the serum-produced transformation than cells carried for shorter periods in vitro. Confirmatory experiments on a second pair of lines supported this conclusion. There are at least two possible interpretations of these observations: 1) FBS selectively favors the growth from a mixed population of those cell types less likely to undergo spontaneous neoplastic transformation, or 2) FBS produces cumulative, long-lasting effects on the cells. The first hypothesis can be tested as soon as we succeed with cloning of nonneoplastic cells in FBS medium. (See #2 below).

f. Heat stability of serum factors.

Results of the previous studies suggested that some component(s) of HS or CS is carcinogenic and some component(s) of FBS suppresses neoplastic transformation. In an effort to characterize these serum factors further, we tested their stability to heating to 55°C for 30 minutes. We found all heated serums (HS, CS, and FBS) still active and comparable to unheated controls in their influence on neoplastic transformation.

2. Establishment of clones from single cells during early growth in vitro and growth of cell colonies at low O_2.

Long-term clones from critically isolated single cells are needed for two groups of studies: 1) for meaningful biochemical studies on neoplastic and nonneoplastic cells arising from the same differentiated cell type, and 2) for studies to establish whether the serum effect is an induction phenomenon or the result of cell selection. In

collaboration with Dr. Richter, we found that cloning efficiencies of a number of nonneoplastic cell lines were 0.1%. In order to increase this exceedingly low plating efficiency, we have explored the effects of the following: types of culture medium, concentration and source of serum, method of cell dispersion, O_2 level, etc.

Standard conditions for the optimum formation of clones of mammalian cells usually include 5-10% CO_2 in air. We tested for colony formation in two atmospheres, 5% CO_2:95% N_2 and 5% CO_2:95% air, 9 different rodent cell lines from the NCTC collection. Three different media at various levels of fetal bovine serum were used. No attempt was made to maintain strict anaerobiosis in any of these experiments. Growth as defined by number and size of colonies was always equal or better in the CO_2:N_2 atmosphere than in the CO_2:air. These studies show for the cell lines tested, and more recently for freshly isolated mouse cells, that for optimal plating efficiency, far less O_2 is required than is usually employed.

3. Attempts to induce neoplastic transformation with chemical carcinogens.

We have been attempting to confirm reports in the literature of neoplastic conversion and morphologic alteration induced in Syrian hamster embryo cells by chemical carcinogens. In a collaborative study with Dr. Montes de Oca, we have duplicated the experimental conditions of these published studies as closely as possible. Cells were plated and treated 24 hours later with benz(a)pyrene at 10 ug/ml. Solvent (acetone) and untreated controls were also carried. None of the colonies obtained to date after treatment with carcinogen showed the characteristic of transformed colonies as reported. We have compared Syrian hamster embryo cells from animals with and without the Graffi agent. Embryo cells from the former exhibit the morphologic alteration even without the carcinogen, and in assays to date carcinogen treatment does not enhance this alteration. We have been testing a number of variables that we think may influence this assay system.

Proposed Course:

These three projects will be continued to attain the objectives listed.

Honors, Awards, and Other Activities:

Member, Advisory Committee of the Animal Cell Culture Collection of the American Type Culture Collection
Member, Board of Directors, American Association for Cancer Research.
Member, Editorial Advisory Board of Cancer Research.
Member, Editorial Board for "IN Vitro".
Assistant Editor for "In Vitro".
Invitation to speak: International Cancer Congress, Houston, Texas, May 1970.

Publications:

Jackson, J.L., Sanford, K.K., and Dunn, T.B.: Neoplastic conversion and chromosomal characteristics of rat embryo cells in vitro. J Nat Cancer Inst 45: 11-23, 1970.

Serial No. NCI-3213, Project 2 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project 2, Title: Manifestations of neoplastic transformation in vitro.

Principal Investigator: K. K. Sanford.

Other Investigators: S.L. Handleman, M.L. Hursey, and C.U. Garrison.

Cooperating Units: W.T. Hall, Electro-nucleonics Laboratories, Inc. Bethesda, Md.

Project Description

Objective: To identify any characteristics of cells in culture that may be directly or indirectly associated with their neoplastic transformation in vitro.

Methods Employed:

At various stages of in vitro growth, cultures of hamster and mouse embryonic cells were examined for cytologic changes and for neoplastic transformation by coverslip preparations and animal assay, respectively.

Major Findings and Significance:

Since one of the projects referred to in last year's annual report was published this year, we will summarize this first. The collaborators in this study were listed in last year's report and included B.E. Barker, R. Parshad, B.B. Westfall, M.W. Woods, J.L. Jackson, D.R. King, and E.V. Peppers.

1. Neoplastic conversion in vitro of mouse cells: Cytologic, chromosomal, enzymatic, glycolytic, and growth properties.

To obtain mouse cells free of any contaminating virus that could be implicated in neoplastic conversion in vitro, we used the germfree ALBM-2 mouse. Cell lines gave negative tests for 11 murine viruses, leukemia virus complement fixing antigens, and recoverable leukemia virus; also from electron microscope examination the cells appeared to be free of C-type particles. With cells derived from a litter of minced embryos we made the following observations:

a. These presumably virus-free cells underwent spontaneous neoplastic transformation, and the type of serum supplement to chemically defined medium influenced the frequency of occurrence and the latent periods for development of tumors.

b. We confirmed our previous conclusion that under the culture conditions used, an experienced cytologist, by following the cytologic changes, could diagnose accurately the neoplastic conversion of cells in vitro. EM studies on sectioned cells showed that the cytoplasmic basophilia of the neoplastic cells was associated with more numerous ribosomes occurring free or clumped, as well as coating numerous surfaces of rough ER, whereas in nonneoplastic cells increasing in numbers at approximately the same rate, the rough ER had greatly dilated cisternae and the cytoplasm contained relatively fewer ribosomes scattered singly or in clumps. From the rather consistent increase in cytoplasmic basophilia associated with increased densities of ribosomes and polysomes in the cytoplasm of the neoplastic cells, we might infer that protein synthesis is more active in the neoplastic as compared with the nonneoplastic cells, even when growing at similar rates in vitro. Further studies on protein synthesis and mitotic rates in neoplastic and nonneoplastic cells from one cell would be desirable to firm this conclusion. Other ultrastructural features of the nonneoplastic as compared with the neoplastic cells correlated well with the stained preparations as viewed by light microscopy.

c. These mouse cells showed a greater stability of karyotype during growth in vitro or as tumors in vivo than observed in C3H mouse cells. Certain cell lines maintained a high frequency of diploid or near-diploid cells for more than 18 months in vitro, and tumors from implants of these exhibited an equally high proportion of such cells. From these observations, we conclude that a shift from diploidy to heteroploidy is not essential for spontaneous neoplastic conversion in vitro. As with C3H mouse cells, the frequency of abnormal chromosomes was higher in the cell line carried on HS supplement and maintained without freezing than in the parallel line on FBS. Although specific chromosomal abnormalities tended to appear in the cells in vitro at variable intervals before neoplastic conversion, the random distribution of chromosomal markers in the various tumors and their complete absence in others revealed no structural abnormality that could be strictly correlated with neoplastic conversion.

d. As part of our effort to analyze the mechanism of serum effect, we examined certain enzyme activities that might be influenced by the serum supplement used. A second objective was to detect any enzyme changes that might correlate with neoplastic conversion. The 3 enzymes chosen were alkaline phosphatase, acid phosphatase, and arginase. We extended to embryo cells the observation, made previously on adult and neonatal mouse cells, that alkaline phosphatase activity declines during long-term culture, whereas high acid phosphatase activity is maintained. No changes in enzyme activity could be correlated with neoplastic conversion. Arginase activities were, in general, maintained at higher levels in the cells cultured in FBS-supplement than in those in HS-supplement. Although possibly the type of serum influences arginase synthesis, these activities observed with the two serums presumably result from selective propagation of different cell types. FBS may support the survival and growth of a broader spectrum of the cell types originally present in the embryo mince. If so, the mechanism by which FBS, as compared with HS, delays or inhibits neoplastic conversion may be indirect by supporting the growth of cell types less susceptible to neoplastic conversion. Further studies on clones are necessary to resolve this point.

e. Transformed neoplastic cells reputedly attain a higher saturation density than control normal cells. This conclusion was based largely on experiments in which virus-transformed cells were compared with control cells, many of which were newly isolated and failed to continue progressive growth in culture. Certain tumor viruses also induce a proliferative response that may or may not be associated with neoplastic conversion. For these reasons, we tested whether neoplastic cells attain a higher saturation density than nonneoplastic cells when the latter have adapted to long-term culture and the neoplastic cells have been spontaneously converted. Saturation density, or the maximum population attainable at confluency or at the stationary growth phase under a given set of conditions, depends on rates of mitosis and necrosis, cell layering, and average area of cell spread.

We found that with increasing time in culture, the cells acquired higher rates of population increase and higher saturation densities, presumably through cell selection and/or adaptation. Cells that had undergone neoplastic conversion had growth rates and saturation densities similar to those of nonneoplastic cells when tested after identical periods in vitro in the type medium to which the cells were adapted. When compared in the same types of medium, transfer to FBS seemed to stimulate the neoplastic cells, and their saturation density was also slightly higher in HS-supplemented medium.

In all assays the neoplastic cells adhered less tightly to the glass substrate and tended to clump, detach, and slough at saturation density.

f. Another objective was to detect any changes in the glycolysis of the cells that could be correlated with their neoplastic conversion in vitro. The rates of aerobic glycolysis appeared to be inversely related to cell density, as reported previously, and were not significantly higher for the neoplastic than the nonneoplastic cells. However, this method of determination during relatively prolonged active growth probably does not measure the full glycolytic potential of the cells as accurately as the short-term (1 hr) manometric method under anaerobic conditions. The cells maintained in chemically defined medium with FBS for 107-334 days were either nonneoplastic or had long tumor-latent periods and showed no progressive increase in anaerobic glycolytic activity by the manometric method. However, the cells with HS supplement underwent neoplastic conversion, produced tumors with short latent periods, and their rates of anaerobic glycolysis tended to increase with time in culture. The cells in FBS-supplement were more sensitive to glycolytic inhibition by a specific anti-insulin (anti-hexokinase) inhibitor than were cells with HS-supplement when tested in their respective growth media. The data are consistent with the view that FBS relative to HS may help to maintain this hormonal control mechanism.

g. Of the many changes observed in these presumably virus-free embryo cells during their adaptation to long-term culture, only three appear to be associated with neoplastic conversion: the progression of cytologic changes, the growth-pattern change characterized by reduced adhesion of neoplastic cells to substrate and slightly higher saturation density, and the change in glycolytic capacity and response to inhibitor.

The increase in saturation density has usually been interpreted as resulting from the loss of contact inhibition of mitosis. Conversely, the cessation of growth in confluent monolayer cultures of nonneoplastic cells was interpreted as resulting from cell contact. From the observations of the present study we suggest a different interpretation of this growth-pattern change. Most cells derived from fixed tissues in vivo require a solid surface substrate which allows membrane spreading and the resultant large increase in cell surface exposed to medium. In a confluent culture of such cells, mitosis may be inhibited, not because of cell contact but because of lack of available surface substrate. The tendency of the neoplastic cells to retract from each other and from the substrate and to grow on each other or even in suspension suggests that they are less dependent on a surface substrate for membrane spreading. Whereas with the nonneoplastic cell in dense culture the rate of cell division may depend on the area of surface substrate available for cell spreading, with the neoplastic cell this requirement appears to be less critical. This reduced substrate dependency, rather than the hypothesis of loss of contact inhibition of mitosis, may account for the growth-pattern change observed in the neoplastic conversion of cells under certain culture conditions in vitro.

2. Cytologic manifestations of neoplastic transformation in vitro.

Presently the only conclusive assay for neoplastic transformation of cells in vitro is to determine their capacity to grow as malignant neoplasms on implantation into compatible hosts. Such assays are not only time-consuming but also require large numbers of cells, are usually limited to cells from inbred animals, may be complicated by possible immunologic changes during culture, and are frequently difficult to interpret especially if negative. Reliable criteria of neoplastic transformation that can be applied directly to populations of cells or even to individual cells in culture are needed. Such criteria might also provide an insight into the changes occurring in the cells during neoplastic conversion in vitro.

As indicated in project 1, we have continued to correlate results of in vivo assay with cytologic changes in the cells during growth in culture. We have attained considerable precision in diagnosing neoplastic transformation in mouse cells grown under certain culture conditions. Hamster embryo cells have presented some problems. With 4 long-term cell lines of control cultures and those exposed to chemical carcinogens, we have attempted with coded samples to establish a more quantitative evaluation of neoplastic state. Results from this extensive study are currently being evaluated.

3. Mucopolysaccharides of neoplastic vs. nonneoplastic cells.

Some observations in the literature suggested that transformed cells have an increased mucopolysaccharide deposit at the cell surface (Cancer Res. 29: 925, 1969). Since these observations were based on a comparison of freshly isolated normal cells and long-term lines of transformed cells, it seemed desirable to compare a pair of neoplastic and nonneoplastic cells of common origin that had been carried for more equivalent periods in culture and grew at similar rates. In collaboration with Dr. Hall these were examined by EM after ruthenium red staining. We found no marked differences

between the neoplastic and nonneoplastic lines in measurable thickness of mucopolysaccharide deposit.

Proposed Course:

The second project will be completed for publication. The EM studies will be continued on cells treated with cyclic AMP.

Publications:

Sanford, K.K., Barker, B.E., Parshad, R., Westfall, B.B., Woods, M.W., Jackson, J.L., King, D.R., and Peppers, E.V.: Neoplastic conversion in vitro of mouse cells: Cytologic, chromosomal, enzymatic, glycolytic, and growth properties. J Nat Cancer Inst 45: 1071-1096, 1970.

Serial No. NCI-3234A SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Primary biochemical alterations leading to neoplastic conversion.

Principal Investigator: R.R. Gantt

Other Investigators: V.J. Evans, K.K. Sanford, F. Montes de Oca, G.H. Smith, and K.J. Stromberg.

Project Description:

A program to elucidate the primary chemical alterations which are necessary biochemical changes leading to neoplastic transformation.

Objectives:

To apply the various biochemical techniques to a system developed in the Tissue Culture Section (see NCI-3211) in order to identify and understand the chemical events leading to neoplastic transformation. There are three immediate objectives:

1. To identify primary changes in the methylation of nucleic acids during neoplastic transformation and other basic cellular alterations such as pre-neoplastic changes and viral infection.

2. To identify the causal agent (or agents) responsible for the dramatic difference between tissue culture media containing fetal bovine serum and media containing horse serum (see NCI-3212 and NCI-3213).

3. To compare in a general way the physical and chemical difference in the membrane components of some RNA tumor viruses and their host cells.

Methods Employed:

1. In order to identify primary changes in the methylation of nucleic acids, differences are first being established before and after neoplastic conversion. These differences will then be correlated with conversion time and with the method of conversion (i.e., viruses, chemical carcinogens, or the horse serum-fetal bovine serum system). These differences are monitored by double isotope co-elution chromatography and enzyme analysis.

2. Identification of the agent (or agents) responsible for the difference in fetal bovine serum and horse serum is being carried out by a series of parallel fractionation and appropriate assay of these fractions. This

consists of ultrafiltration through a collodion membrane, Sephadex gel filtration, ammonium sulfate precipitation, and subsequent tests for their ability to transform cells or to protect the cells from transformation.

3. Two plant agglutinins, concanavalin A and a wheat germ glycoprotein, are known to react with specific sites on membranes. The availability of these sites seems to vary and may be related to the ability of cells to grow over one another. Since the RNA tumor viruses have a membrane envelop at least partially derived from the host cell, these specific agglutinins will be used to see if the specific sites they react with are available on the membrane surface of the virus.

Major Findings:

1. Nucleic acid methylation.

a. The isolation of mouse satellite DNA which has been labeled in the methyl group of 5-methylcytosine has demonstrated two things. First, that both strands of the satellite DNA are equally methylated, and second, that when the specific activity of main band DNA is compared with the specific activity of satellite DNA in the paired neoplastic and nonneoplastic cell lines, there is no difference in methylation.

b. A specific RNA methylase has been found associated with the avian myeloblastosis virus. This enzyme transfers a methyl group from S-adenosylmethionine to the N^2 position of guanine using transfer RNA as an acceptor. This activity has also been detected in the Rous sarcoma virus.

2. The results of long-term experiments involving serum fractionation have shown that the factor(s) implicated in spontaneous malignant conversion is in the small molecule fraction (molecular weight less than 5000).

3. No major results have yet been found on this project because of insufficient qualified personnel.

Significance and Proposed Course of Investigation:

1. a. The finding that both strands of mouse satellite DNA contain equal amounts of 5-methylcytosine in spite of the large difference in cytosine content between the strands is consistent with the idea that methylation of DNA is required for DNA to serve as a template. The importance of this idea is that aberrant methylation of DNA could plausibly lead to deletion of regions of DNA resulting in somatic mutations. This then provides a molecular scheme which would unify under one mechanism the carcinogenic effects of compounds as diverse as ethionine, the cyclic hydrocarbons, and alkylating agents. An important aspect of this mechanism is that there are several available approaches to test it. For the time being, however, this project will be dropped because of insufficient resources.

b. The discovery of a specific RNA methylase associated with two oncogenic viruses is very important in view of the large body of circumstantial evidence implicating aberrant methylation with malignant conversion. The proposed course of investigation is as follows:

(1) Purify and characterize the enzyme for two reasons: first, to produce antibodies so that single cell assays can be developed for its presence; and second, establish the neighboring nucleotide specificity to assess whether the enzyme, though present, is actually active because of the known presence of specific inhibitors.

(2) Localization of the activity, i.e., is it in the core or in the volume between the core and envelope?

(3) Assess the role of the specific methylase in the virus life cycle.

(4) Look for the specific presence of N^2-methylguanine in the paired neoplastic and nonneoplastic cell lines.

2. The discovery that the active factor which either induces or accelerates the spontaneous neoplastic conversion has two important aspects:

a. It may be possible to remove this factor from horse serum and maintain cell lines in vitro for very long periods of time without spontaneous conversion. Already cells have been grown in horse serum where the small molecules have been removed and after two years conversion has not occurred, while lines in which the small molecular weight component has been added back conversion has long since occurred.

b. Isolation and characterization of the small molecular weight component may result in greater insight into the mechanism of spontaneous malignant conversion.

Besides the obvious courses of continuation, it may be of value to attempt to adapt nonmalignant cell lines to chemically defined media by initiation and growth in large molecule fractions followed by gradual depletion of serum components as has been done in the past. Though a long shot, it may result in nonmalignant cell lines in truly chemically defined media which would be a decided technical advance.

3. Though this program is in the first stages of development, its ultimate end is to understand the virus host cell relationship. For example, since it has been shown that these membrane sites are unmarked when a normal cell goes through mitosis, and cell division is required for infection, there may be a viral requirement for the unmasking of these sites during infection to promote a cell membrane-viral membrane interaction. Experiments are planned to test this possibility.

Honors and Awards:

Presented our work at an international colloquium on "Transfer RNA and Transfer RNA Modification in Differentiation and Neoplasia," Oct. 1970.
Invitation to present our work at the Cold Spring Harbor Laboratory meeting from May 29 to June 1, 1971.

Publications:

Quinn, C.E., Gantt, R.R., and Evans, V.J.: Comparison of methyl-labeled tRNA in a neoplastic cell line with a paired nonneoplastic control. Exp & Mol Path 13: 231-241, 1970.

Gantt, R.R.: In vitro tRNA methylation of paired neoplastic and nonneoplastic cell cultures. Cancer Res 31: 000-000,1971.

Price, R.M., Gantt, R.R., and Evans, V.J.: Effect of fractions of horse and fetal bovine serum on neoplastic conversion of C3H mouse cells in tissue culture. In Vitro 6: 000-000, 1971.

Serial No. NCI-3257 NONE

1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Factors influencing the growth and neoplastic conversion of cells in vitro.

1. Influence of glycine peptides upon cells in vitro.
2. Efficiency of commercially available peptones as cell growth stimulants and/or substitutes for serum.
3. Evaluation of some organic buffers for cells in vitro.
4. The development of a homeostatic cell culture system.
5. Serum hamster cells exposed to benzpyrene. (Refer to Serial No. NCI-3213, project 1)

Principal Investigator: W. G. Taylor

Other Investigators: V. J. Evans, K. K. Sanford, R. A. Dworkin, F. M. Price, and E. V. Peppers.

1. Influence of glycine peptides upon cells in vitro.

Objective: To elucidate the influence, if any, of low molecular weight glycine peptides, a large component in peptone dialysate, upon the growth and survival of tissue culture cells in chemically defined medium.

Methods Used:

Five glycine peptides, containing two through six amino acid residues (Schwarz-Mann Bioresearch, Inc.) were prepared as an aqueous solution at five different millimolar concentrations (0.045-0.72 mM). These were used to supplement medium NCTC 135; Earle's L cell adapted to chemically defined medium (i.e., strain 2071) was used as a test model. Replicate cultures were prepared by pooling cells from several T-60 flasks and dispensing them in 3 ml aliquots with a modified Cornwall automatic syringe pipette. Eighteen to twenty-four hours later, the planting medium was decanted and 4 ml of the appropriate culture medium added to each of 6-10 T-15 flasks. The cultures were incubated at 37.5°C for 3 days at which time some cultures were evaluated for cell population increase; the experimental growth medium was decanted from the remaining culture and 3 ml of new medium added. The latter cultures were usually evaluated for cell population increases after five days incubation in the peptide-containing medium. An increase in cell number was used as an index of stimulation; cell counts were performed with a Coulter Model B Counter.

Sublines (designated 6797-6801) of strain 2071 were developed by continuously subculturing populations of 2071-like cells in 0.72 mM glycine peptide supplemented NCTC 135. After at least 20 subculturings (transfers), these were evaluated for an increased or decreased neoplastic potential by IM injection into C3H mice. In addition, the dose responsiveness of these sublines was evaluated with peptide concentrations of 0.09-0.72 mM in glycine free NCTC 135.

Major Findings:

Representative data from two different experiments are tabulated below, and are expressed as the "fold increase" of cells (i.e., the ratio of the total cell number after incubation to the cell number first exposed to the peptide-supplemented medium).

0.72 mM peptide	Experiment A[1] 4 days[2]	Experiment A[1] 6 days[2]	Experiment B[1] 5 days[2]
glycylglycine	3.59	8.49	2.90
triglycine	4.41	7.85	2.42
tetraglycine	5.13	8.95	3.14
pentaglycine	4.12	7.08	1.05
hexaglycine	3.40	8.43	1.98
Control 135	4.19	7.66	2.28

[1]Cell number at 24 hrs: Exp. A, 3.43×10^5; Exp. B, 1.45×10^5.
[2]Incubation period with experimental medium.

Thus, only tetraglycine seemed stimulatory for strain 2071. Reduced concentrations of this and the other peptides yielded equivalent cell populations, however, and though the most remarkable stimulation was observed with low cell inocula, cell survival rather than active proliferation is suggested when the control data from the two experiments are compared.

When the sublines of 2071 carried in 0.72 mM peptide were injected into C3H mice, solid tumors grew at the site of injection in all cases. These peptides do not alter the neoplastic potential of strain L. Titration of the biological efficiency of these peptides in vitro in the absence of glycine suggests that, with the possible exception of tetraglycine, these peptides are poorly metabolized; alternatively, strain 2071 may have developed a subtle glycine or serine requirement not heretofore observed (Evans, V.J., et al., Am. J. Hyg. 68: 66-73, 1958).

Significance and Proposed Course: Earlier studies on a rabbit cell line had indicated that peptone dialysate (PD) was stimulatory to growth when a medium free of serum was used. This particular peptone dialysate showed the presence of a large amount of glycine peptide. Though tetraglycine was somewhat stimulatory for 2071 cells, the evidence is that it is not, at least for 2071 cells, highly stimulatory. Assessment of the effect of these peptides

on the rabbit cell line in which the peptone dialysate had been found stimulatory is planned to determine if there is a strain difference or if the glycine peptides are or are not the stimulatory factor in the peptone dialysate. Determination of the stimulatory factors in PD is part of an endeavor to substitute for serum, with its complicating actions, in initiating and propagating many strains of cells in vitro. Should the glycine peptide prove stimulatory for the rabbit cell line, the mechanism of activity (presumably "protective") will be further studied.

2. Efficiency of commercially available peptones as cell growth stimulants and/or substitutes for serum.

Objective: Bacto-peptone (Difco, Inc.) is used widely as either a supplement in serum containing culture medium, or as a substitute for serum in chemically defined medium. The purpose of this investigation was to evaluate other commercially available peptones for their biological efficiency in vitro.

Methods Employed:

Peptones from several sources were prepared in Earle's saline as 5-20% stock solutions and filter sterilized. A biuret test was performed on each stock solution using the Accu-Stat Blood-Chemistry Analyzer (Clay Adams), and the volume of peptone employed/100ml growth medium adjusted to be equivalent to that of fetal bovine serum (4.2 gm %).

All other procedures are identical to those described in section 1 above. All cultures were grown in NCTC 135 or peptone supplemented NCTC 135.

Major Findings: Typical results are tabulated below.

Supplement	Source	Fold increase at[1] Day 3	Day 5
None	--	2.62	4.16
10% FBS	Flow	2.50	3.03
Trypticase	BBL	1.35	1.11
Myosate	BBL	Not done[2]	
Acidicase	BBL	Not done[2]	
Peptic Peptone	NBC	1.64	1.62
Peptone T	NBC	1.35	1.23
Tryptic Casein	Colabs	1.25	0.58
Witte's		1.63	1.16
Beef Peptone	Mann	Not done[2]	
Bacto-Peptone	Difco	0.64	0.67

[1]Fold increase defined as the ratio of cell numbers after 3 or 5 days incubation to that number present when the experimental medium was first added.

[2]Preliminary studies indicated that no growth occurred in the presence of these peptones.

These results suggest that the peptones evaluated were not beneficial for 2071 (strain L adapted to chemically defined growth medium).

Significance and Proposed Course:

In each of the experimental series performed, the cultures receiving 10% FBS unexpectedly proliferated to approximately the same degree as those in 135 alone. This, coupled with unpublished evidence that no significant difference between types or sources of peptones is observed in cell lines adapted to continuous growth in Bacto-peptone, suggest either: (i) strain 2071 is unable to benefit from various peptones (or serum) due to its extended growth in chemically defined growth medium (CDM); (ii) that these peptones are biologically toxic for cells adapted to CDM.

Further work, therefore, includes retesting the peptones in cultures of rabbit heart cells adapted to growth in peptone, and removing any "toxic" components from crude peptone by the conventional methods.

Normal serum is known to contain enzymes, hormones, natural antibodies, nonspecific cytotoxic entities, and microbial contaminants. Thus, an efficient substitute for serum in tissue culture medium is required. This study represents an evolution of such a substitute.

3. Evaluation of some organic buffers for cells in vitro.

Objective: Since the introduction of zwitterion organic buffers by Good (Biochemistry 5: 467, 1966) a limited number of studies have been undertaken to evaluate their usefulness for tissue culture systems. The purpose of this study is: (i) to evaluate the usefulness of such buffers in chemically defined medium (CDM), and simultaneously study the physiological requirement for bicarbonate by cells in vitro, and (ii) to evaluate the role, if any, of bicarbonate in in vitro carcinogenesis.

Methods Employed: HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was prepared as an aqueous 0.25 M solution (10x). It was used to buffer NCTC 135 containing 2.2, 1.1, and 0.0 gm/liter. In the latter case, two different phosphate buffered salines were used. Whenever bicarbonate was contained in the growth medium, duplicate sets of cultures -- gassed with humidified 10% CO_2/air and ungassed -- were prepared. The cells employed were Strain L adapted to continuous growth in CDM (strain 2071). All other procedures were identical to those described in Section 1.

Major Findings: Because the cell inoculum number was reduced to less than 2×10^5 cells/flask, a somewhat lessened population was observed after 3 and 5 days incubation in the experimental medium. These results are valid, however, and a typical set of data is compiled below.

gm bicarbonate per liter 135[a]	Employed HEPES	Gassing	Fold Increase[b] Exp. A day 3	Exp. A day 5	Exp. B day 3	Exp. B day 7
2.2	-	+	1.63	1.93	2.13	3.23
2.2	+	+	1.57	1.68	1.86	3.50
2.2	+	-	1.39	1.47	2.16	4.21
1.1	+	+	1.52	2.56	1.25	1.54
1.1	+	-	1.47	1.64	1.86	2.95
0.0[c]	+	-	1.46	1.72	1.42	1.04
0.0[d]	+	-	1.34	1.51	1.46	1.03

[a]NCTC 135 contains 2.2 gm sodium bicarbonate per liter of medium.

[b]Seed inocula: Experiment A: 1.59×10^5 cells/flask
Experiment B: 1.20×10^5 cells/flask

[c]Dulbecco's PBS used.

[d]Puck's PBS used.

Significance and Proposed Course: In general the above data suggest that strain 2071: (i) can proliferate and survive in medium containing reduced amounts of sodium bicarbonate, (ii) is not adversely affected when HEPES is added to CDM, and (iii) requires more bicarbonium ion than is produced by the catabolism of glucose, as evidenced by the cell maintenance in medium prepared in PBS. The latter was not unexpected (Harris, M., J. Exptl. Zool. 125: 85, 1954; Swim, H.E. and Parker, R.F., J. Biophys.& Biochem. Cyto. 4: 525, 1958). To obviate this, we intend to reduce the bicarbonate level as far as possible and supplement the medium with Kreb's cycle intermediates such as pyruvic and citric acid. Attempts will be made to initiate cells in bicarbonate-free medium to evaluate their spontaneous neoplastic conversion.

4. Development of a homeostatic cell culture system.

Objective: Current cell culture media and techniques subject the cell to a myriad of physical and chemical variations. The most obvious are the pH fluctuations observed between seeding and monolayer formation, and the depletion of nutrients such as glucose and essential amino acids over the same period. This constant physio-chemical variation undoubtedly influences the biological character of the cell population.

Heretofore, only one laboratory has made significant technical advances in the control of physio-chemical variations for large cell populations (Kruse, P. et al., In Vitro 6: 75, 1970). All of their reported work, however, was performed with established cell lines.

Our objectives are to develop homeostatic cell culture techniques and in so doing evaluate the influence of nutrients, trace metals, pH, pO_2, pCO_2, temperature, etc., upon spontaneous neoplastic transformation, chromosomal aberrations, etc. This will be done with freshly explanted tissue.

Methods Employed: These are all in the developmental stages.

Significance and Proposed Course: Because all current investigation is carried out under conditions permitting vast variation, the influence of physio-chemical fluctuation upon in vitro carcinogenesis cannot be ascertained until homeostatic culture techniques are available. Our attempts to develop such systems have been unsuccessful thus far due to bioengineering problems which we are now attempting to resolve.

Honors and Awards:

Associate membership, Sigma Xi.
Nominated for the Milan Novak Award, given to the most outstanding graduate teaching assistant (1969-1970) in the Department of Microbiology, University of Illinois at the Medical Center, Chicago, Illinois.

Publications:

1. Pumper, R.W., Fagan, P., and Taylor, W.G.: Trypsin sensitivity of mammalian cells grown in a serum-free medium. In Vitro 6: 266-268, 1971.

2. Rutzky, L.P., Taylor, W.G., and Pumper, R.W.: In vitro stimulation of rat liver cells by homologous partial hepatectomy serum. In Vitro 7: 000-000, 1971.

3. Taylor, W.G.: (Abstract) Isolation of a growth substance for mammalian cells cultured in vitro. In press, Dissertation Abstracts, June, 1971.

4. Taylor, W.G., Taylor, M.J., and Pumper, R.W.: (Abstract) Separation and preliminary analysis of a growth substance from Bacto-peptone. Abstract #125, 22nd meeting of Tissue Culture Association, June, 1971.

Laboratory of Cell Biology

SUMMARY

July 1, 1970 - June 30, 1971

Lloyd W. Law

Some highlights of achievements in the Laboratory of Cell Biology:

1. It has been shown following solubilization and partial purification of membrane histocompatibility antigens (H) that fractions are obtained in which separations of H-2 from non H-2 may be achieved - using A strain ($H\text{-}2^a$) spleen cells. The serologic specificities - detected by inhibition of ^{51}Cr release and transplantation antigens - detected by skin graft rejection (histologic epithelial survival) appears to reside on the same molecule and in all probability are the same antigens. It has also been shown that the fraction F_2 (obtained by G-150 Sephadex fractionation) has enhancing ability using an allogeneic tumor graft system. This indicates that the purification steps achieved to the present preserve all of the antigenic specificities determined by the $H\text{-}2^a$ locus. Further steps in the purification procedure are in progress with in vitro monitoring using inhibition of cytotoxicity and immunogenic testing in the CBA → A skin graft test.

2. There has been developed a simple, rapid, objective method for studying membrane bound TSTA. ^{51}Cr release assay has proved effective in studying the TSTA of specially selected and adapted tumor cells induced by SV 40 virus. The A strain SV·A/LN cells and BALB/c strain MKSA cells both contain strong SV·40 specified TSTA. One of those MKSA grows progressively in syngeneic hosts so that in vivo immunogenicity studies are in progress. Antisera to to SV·A/LN cells are cytotoxic to both tissue-culture adapted target cells and to hamster cells (induced SV 40 neoplasms) known to contain TSTA. Crude soluble membrane peparations have been shown to inhibit the cytotoxicity of the specific antibody - thus allowing us to procede with further steps in the purification of tumor antigens. The advantage of using the mouse system in purification of tumor antigens lies in the fact that the presence of H-2 and non H-2 specificities provide positive markers in the purification steps and allow distinctions to be made between specific tumor antigens and those specified by H-2 and non H-2 genetic loci.

3. A method has been developed for the quantitative measurement of α-Fetoprotein. Routinely the new technique will measure as little as 350 nanograms and with a modification using radioactive antiserum and autoradiography, levels as low as 20 nanograms α - FP may be measured. The technique is being used to quantitate serum levels of patients with hepatomas and is being applied also in studies of monkey serums in which monkeys are undergoing treatment for hepatocellular carcinogenesis.

4. An antigen common to 5 of 10 pulmonary tumors of man has been studied. This is a soluble antigen similar in some respects to the CEA described by Gold for colonic neoplasms. Though this antigen is not found in normal lung tissue some cross-reactivity has recently been detected in normal tracheal

and esophogeal extracts. This work is being continued upon a larger number of primary lung tumors.

5. Class-specific antiserums have been developed and some of their characteristics elucidated. Anti-γF antiserums are cytotoxic for plasma cells secreting γF but not for other non-γF plasma cell tumors. Thus immunoglobulin receptors at the cell surface appear very specific. Various populations of mouse lymphoid cells have been assayed using class-specific antiserums; only kappa and γM receptors have been demonstrated in certain frequencies for bone marrow, lymph nodes and spleen. Thymocytes were found to be negative for immunoglobulin determinants.

6. Studies are continuing on intracisternal A-particles which are found initially in plasma cell tumors but have now also been found in normal as well as neoplastic tissues in five strains of mice and their hybrids. The presence of these particles in gonadal tissues suggests that vertical transmission may take place. The protein patterns of intracisternal A-particles are identical in those isolated from 3 different plasma cell tumors. They are deficient in the lower molecular weight proteins found in the murine leukemogenic viruses and they do not contain the PAS-reacting glycoproteins characteristic of MLV - nor is the GS (group specific) antigen of MLV present. These particles are being concentrated and purified in order to determine their biologic functions and relationships to type C particles and the intracytoplasmic A-particles (mammary tumor virus precursors).

7. Previous studies showing differences in aminoacyl-tRNA among different plasma cell tumors have been continued. These patterns appear to be stable, determined characteristics of each tumor and plasma cell tumors differ strikingly from each other - more so than normal tissues - in their aminoacyl-tRNAs (and also most likely in their Leu-tRNAs, Ser-tRNAs and Tyr-RNAs. It is suggested that myelomas (in contrast to normal tissue precursors) differ from each other because the preponderant messenger in each myeloma has one unique structure as opposed to varied populations of messengers in normal cells, and these in myeloma cells are especially differentiated towards secretion of a particular protein.

8. Studies of myeloma proteins in mice for antigen-binding activity continue. Proteins that bind arabinogalactan, a trypsin-sensitive antigen in Salmonella, the influenzal type B polysaccharide and antigen from several bacterial species have been identified recently. A large screen using 60 antigens is being followed. It is hoped eventually to obtain a myeloma protein that may be crystallized and also have antigen-binding activity to a chemically characterized determinant.

9. Myeloma-specific antisera have been obtained through immunization of other strains of mice with BALB/c myeloma proteins of the IgA and IgG classes. These are found to be specific for the immunizing antigen. This type of study provides a tool for determining the number of specific types of immunoglobulins and in searching for similar immunoglobulins in normal mouse antibody that share antigenic specificities with myeloma proteins.

10. MSV, an RNA oncogenic virus, is used to induce neoplastic growths containing membrane-associated TA having the characteristics of H-antigens in rejecting the neoplasms. A complication in studying RNA virus-induced neoplasms results from the constant replication of virus in neoplastic cells thus preventing a distinction between virus-specified new cellular antigens and virion antigens. Through selection a non-producer neoplasm (XM-1; hemangiosarcoma) was selected and shown to contain virus-specific TSTA. Further cloning in vitro of XM-1 shows a highly heterogeneous population of neoplastic cells. Some clones selected and grown in vivo show the continued presence of TSTA in the absence of infectious MSV and MLV and in the absence of "rescuable" MSV. It is thus shown that TSTA induced by RNA oncogenic viruses are analogous to those induced by DNA viruses and that antigenicity of these TSTA results from the presence of viral genetic material present in a form other than that of infectious virus or "defective" virus.

11. Studies continue on the ganglioside changes that occur after transformation of mouse cells in vitro by polyoma and SV 40 viruses. The biochemical change was found to result from inactivation of a key enzyme (N-acetylgalactosamine transferase) necessary for the stepwise elongation of the carbohydrate chain in the gangliosides. This loss of enzyme activity was found not to occur however in RNA virus induced nor in spontaneous transformed neoplastic cells of the mouse.

12. Studies were initiated this year employing the production of hybrid somatic cells to analyze problems in biology and medicine.

a) Collagen synthesis has been analyzed in hybridized cell strains of mouse fibroblasts (collagen+) and rat lymphocytes (collagen -) and shown to occur; 3 other somatic hybrids between (+) and (-) also show collagen synthesis 2 to 5 times greater than in the (+) parent. These results indicate that the negative control of the (-) parent is not operative in these hybrids; also the production of heterokaryons between human fibroblasts (-) and chicken erythrocytes (+) show these to produce the characteristic alpha-2 collagen of the chicken, thus nuclear fusion does not appear necessary for gene "turn on" in the production of collagen at least.

13. Studies of the chemical structures of immunoglobulin molecules reveal important findings:

a) Evidence is now clear for the existence of a single gene controlling the constant region of immunoglobulin light chains in the rabbit. However, rabbit Kappa chains of different allotypic specificities show multiple amino acid differences in their constant region. This argues for the existence of pseudo alleles instead of single alleles. It is unclear at the moment if any antigenic determinant is carried in the variable region of the Kappa chains although complete sequence studies on homogeneous antibodies with different specificities should answer this question.

b) In relation to mouse Kappa and Lambda chains, a striking result is the large amino acid changes accumulated in the Kappa chains versus the identity or limited variability (1 or 2 amino acid interchanges) for

the lambda chains. This is quite species-specific and does introduce further complications of the mechanisms involved in antibody variability. An interesting result however is the preliminary observation that the gamma-As with antibody-like activity to phosphorylcholine have a very limited variability. Definition in the near future of the type and position of this variability both in the heavy and light chains should help in the understanding of antibody specificity.

14. Studies are continuing on the characteristics of MSV(O) detected in a rat neoplasm (MSB-1) induced by MSV(MLV). Preliminary evidence indicates that attempts to infect mouse cells with MSV(O) are successful if MSB_1 cells are first killed with UV-irradiation without influencing the titer of MSV(O). Cloned mouse embryo cells infected with irradiated material now produce MSV(O). Thus the nature of the defectiveness of MSV(O) may be pursued and large amounts of mouse adapted MSV(O) will allow immunologic and physicochemical characterization of this variant virus.

Serial No. NCI-3200

1. Laboratory of Cell Biology
2. Cell. & Tumor Immunol. Sect.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Factors influencing the induction and repression of primary tumors and the growth and inhibition of transplantable neoplasms. (Host and tumor cell variables)

Previous Serial Number: NCI-3200

Principal Investigator: Dr. Lloyd W. Law

Other Investigators:
Dr. E. Appella (NCI-LCB-3229)
Dr. Kenneth Chang (NCI-LCB-5550)
Dr. Peter Wright (NCI-LCB-5551)
Dr. J. McCoy (Not NIH)
Dr. N. Burstein (Not NIH)

Man Years:
Professional - 1 (Dr. L. W. Law
Others - 3 1/2
Total - 4 1/2

Project Description:

1. Role of antilymphocyte serum (ALS) and other suppressants in oncogenesis.

a). Immunosuppression created by ALS in adult (12 week old pathogen-free BALB/c mice established a striking susceptibility to MLV-providing immunosuppression precedes viral infection. Resistance to generalized leukemia may be reestablished in these susceptible adults by adoptive transfer of lymphoid cells (lymph node or splenic origin) from normal syngeneic mice. Timing and number of lymphoid cells in adoptive transfer are critical.

The precise mechanisms concerned with enhanced susceptibility of BALB/c mice following adoptive transfer of lymphoid cells from hyperimmunized syngeneic donors has not been determined. The following additional facts are known:

1). Lymph node cells (2.5 to 6 x 10^7 cells, intravenously) also provide enhancement

2). No differences were detected in in vitro lymphoid cell immune responses between populations from normal and hyperimmunized BALB/c mice

3). Graft versus host reactions were induced equally well in BCF_1 mice by normal hyperimmunized splenic cells

4). Antisera from "hyperimmunized" donors did not produce "enhancement" nor did they prevent the activity of normal lymphoid cells in reestablishing resistance to leukemogenesis in the adoptive transfer experiments

b). In collaboration with Dr. Kenneth Chang (LCB-5550) and Dr. James McCoy (not NIH) it has been found that ALS treatment of adult and BALB/c (or C57 Black) mice prior to infection with MSV(MLV) provides susceptibility to induction of tumors. Antiviral (neutralizing) antibodies parallel the resistance and susceptibility to this virus, high levels of neutralizing antibody appear in those mice resisting oncogenesis. In contrast to the findings of induction of lymphocytic leukemia in BALB/c mice, antisera with high levels of antiviral antibody will completely prevent tumor induction by MSV in ALS-suppressed mice even if administered 2 to 3 days after (MSV(MLV) infection.

c). In collaboration with Dr. Nelson Burstein (not NIH) the long-term effects of 3-day thymectomy were studied employing C57BL/Ka and C57BL x C3Hf/He F_1 mice. Thymectomy in these strains provides a minimal and subtle immunodepression. Effects of thymic removal on longevity and the spontaneous occurrence of hepatomas, mammary tumors and reticulum cell sarcomas were studied. An increase frequency of mammary tumors were observed in BC3H F_1 females - 40% from a frequency of 4% in intact litter mates. The MTV-free mammary tumors appearing as early as 20 months and as late as 33 months ranged histologically from well-differentiated, papillary adenocarcinomas to anaplastic carcinomas that infiltrated into fibrous stroma. Neither the latent period nor frequency of the other commonly occurring neoplasms was influenced. An opposite effect of 3-day thymectomy was recorded from this laboratory upon MTV-induced mammary tumors. Since 2 different antigenic components (TSTA) have been identified on neoplastic cells from mammary tumors (one cross-reactive and MTV-dependent, the other non-cross-reactive and non-MTV dependent) it is our concept that early thymectomy influences different components of the immune response in these 2 experimental situations, assuming that immune deficits and TSTA are major factors in the induction (and repression) of both types of mammary tumors in the autochthonous host.

2. Studies of non-releaser neoplasms.

In order to study tumor antigens (TA) on/in neoplastic cells induced by an RNA oncogenic virus MSV, it has been necessary to select populations of cells not replicating virus. In collaboration with Dr. Kenneth Chang (LCB-5550)

1. Laboratory of Cell Biology
2. Cell. & Tumor Immunol. Sect.
3. Bethesda, Md.

A presumably non-releaser hemangiosarcoma induced by MSV in BC3H F_1 mice was cloned in vitro and the antigenic and neoplastic properties of the clones are now being studied. XM-1 is very heterogeneous. The following clones have been isolated and studied:

H-14	No MSV, No MLV	Neoplastic	TSTA (+)
H-11	No MSV, MLV	Non-neoplastic	TSTA (+)
H-1	No MSV, No MLV	Neoplastic	TSTA (+)
#60	No MSV, MLV	Neoplastic	TSTA (-)

MSV was not rescuable from any of these clones. Tumor #60 resulted from growth of H-14 in vivo and although MSV (or type C particles) was then found to be present, by a sensitive uridine incorporation assay, the antigenicity of this line was lost. Though studies with these and additional lines are being continued, certain conclusions are warranted:

1). The neoplastic state resulting from an RNA virus is not dependent upon the continued presence of the inducing virus either in an infectious form or in a "defective" state.

2). Antigenicity resulting from TA results apparently from the presence of viral genetic material present in a form other than that of infectious virus or "defective" virus.

3. Studies of serologic and immunogenic properties of soluble murine transplantation antigens controlled by the H-2 locus.

As reported last year collaborative studies,with Dr. Ettore Appella (LCb-5551) Dr. Peter Wright (LCB-5551) initiated also with Dr. Samuel Strober who has since left NCI, have yielded important information:

a) The specificities determined by the $H\text{-}2^a$ locus, solubilized and partially purified on a G-150 Sephadex column, were found to be localized to a single peak in the F_2 fraction. Transplantation antigens (H-2) as detected by skin graft rejection (histologic epithelial survival) were also confined to the F_2 fraction. This is the first definitive evidence to show that H-2 transplantation antigens and H-2 serologic specificities are in all likelihood located on the same molecule. Non-H2 transplantation antigens were confined to an F_3 fraction.

b) Further purification is proceeding with the solubilized F_2 fraction with in vitro monitoring using an inhibition of cytotoxicity by monospecific antisera covering most of the H-2^a specificities: 1, 3, 11, 19, 23 and 28. In vivo skin rejection of A → CBA skin grafting will be used.

c) The biologic completeness of the solubilized, purified material was further tested in immunologic enhancement studies. It has been shown that in order to achieve enhancement all antigenic specificities must be present in the enhancing material. It was found that F_2 fraction (H-2) emulsified in Freund's adjuvant at 200 µg/mouse provided enhancing antibody so that a strain A tumor Yaa-Cl grew progressively in CBA strain recipients. The F_2 fraction and adjuvant alone did not provide enhancement. Antisera from mice treated with the F_2 fraction yielded cytotoxic activity (> 1:128) whereas adjuvant-control antisera did not (1:8). Further work is in progress to determine in vivo and in vitro the specificity of this response.

4. Tumor antigens: Attempts at solubilization and purification.

Collaborative work with Dr. Ettore Appella (LCB-3229) and Dr. Peter Wright (LCB-5551) concerns attempts to further characterize virus-specific tumor transplantation antigens (membrane bound TSTA) with the ultimate purpose of obtaining soluble, purified TSTA.

It was necessary to obtain populations of neoplastic cells bearing strong TSTA, to adapt these to tissue culture and to select those that provided sensitive, quantitative in vitro assays. The SV40 specified TSTA on AL/N cells and MKSA (A strain and BALB/c strain respectively provided good assay systems for inhibition of cytotoxicity, using hyperimmune antisera. Antigens prepared from tissue-culture grown SV·AL/N cells (neoplastic cells bearing SV40 specified TSTA) were able to inhibit the cytotoxicity of antisera as measured by release of ^{51}Cr label. Crude membrane fractions of the neoplastic cells were also effective, thus allowing us to pursue with solubilization.

The use of ^{51}Cr-labelled target cells to detect humoral antibodies and their inhibition by antigen is a simple, rapid, objective, reproducible method that allows one to quantitate concentrations of TSTA in neoplastic cells..

1. Laboratory of Cell Biology
2. Cell. & Tumor Immunol. Sect.
3. Bethesda, Md.

Publications:

Strober, Samuel and Law, Lloyd W.: Initiation of antibody responses by different classes of lymphocytes. IV. Lymphocytes involved in the primary antibody response to a hapten protein conjugate. Immunology. (in press).

Wright, Peter W., and Law, Lloyd W.: Quantitative in vitro measurement of SV40 tumor-specific antigens (TSTA's). Proc. Nat. Acad. Sci. (in press).

Strober, S., Appella, E., and Law, L. W.: Characterization of the immunogenic and serological activity of soluble H-2 transplantation antigens in the mouse. Transplantation Proceedings. (In press).

Law, L. W., and Chang, S. S.: Effect of antilymphocyte serum (ALS) on the induction of lymphocytic leukemia in mice. Proc. Soc. Exper. Biol. Med. 136: 420, (1971).

Strober, S., Appella, E., and Law, L. W.: Serological and immunogenic activity of soluble mouse transplantation antigens controlled by the H-2 locus. Proc. Nat. Acad. Sciences 67: 765-772, 1970.

Steinberg, A. D., Law, L. W., and Talal, N.: The role of NZB-NZW F_1 thymus in experimental tolerance and immunity. Arthritis and Rheumatism 13: 369-379, 1970.

Serial No. NCI-3229

1. Laboratory of Cell Biology
2. Cell. & Tumor Imm. Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical structure of immunoglobulin molecules

Previous Serial Number: NCI-3229

Principal Investigator: Dr. Ettore Appella

Other Investigators: Dr. R. Mage (NIAID-148)
Dr. A. Chersi (Visiting Scientist, NIAID)

Man Years: Total - 1 3/4
Professional - 1
Other - 3/4

Project Description:

1. Rabbit light chains

The allotypic markers of the kappa-type light chains of rabbits, b4, b5, b6 and b9 behave as single "Mendelian" allelic genes. Chemically they appear to reflect multiple amino interchanges which are within the constant region of the molecule. The majority of these light chains contain 7 half-cystine residues instead of 5. By digestion with pepsin and chymotrypsin cysteine-containing peptides have been isolated and purified. There is no evidence so far of any amino acid substitution related to allotypic specificity present in the variable region of kappa chains.

By the use of a Beckman Sequencer 890, the amino-terminal sequences of light chains of different b-locus allotypes have been examined as far as residue 23. When aligned with human and murine kappa chains, it is apparent that there are three chain lengths which have been designated V_{KI}, V_{KII} and V_{KIII} subgroups. The majority of light chains carrying b4 and b5 allotypes have the V_{KII} length; b6 light chains have V_{KI} length and b9 light chains are unique in having valine at position 1 for the V_{KIII} length instead of isoleucine. These observations suggest that V_K genes can be associated with any of the four b-locus allotypes but expressed in different proportions depending upon the allotypes. Continuing investigation of the amino acid sequence of homogenous rabbit antibodies available at the present time should provide data on the exact chemical localization of the b-locus allotypes and the relationship of hypervariable regions to antibody specificity.

1. Laboratory of Cell Biology
2. Cell. & Tumor Imm. Section
3. Bethesda, Md.

2. Mouse light chains

Ten kappa chains produced by different plasma cell tumors induced in BALB/c have been analyzed in the Beckman Sequencer. A large degree of variability has been found in the first 23 amino acids. A comparison with published sequences has been made and additional subgroups have been found. The mouse kappa chains have more prototype sequences than their human counterparts (18 as opposed to 3) and one possible explanation is that the myeloma process reflects a greater kappa chain diversity in mouse as opposed to man. The λ:κ ratio in mouse is 3:97 as compared to 1:2 in man. Studies of mouse lambda chains in our laboratory do not as yet reveal a pattern of subgroups. Perhaps the mouse has had to expand its V_κ gene population to compensate for a lack of V_λ genes.

3. Rabbit heavy chains

The structural basis for the A14 and A15 allotypic specificities of rabbit immunoglobulin-G have been investigated. Fc fragments were prepared from homozygous rabbits and each tryptic peptide isolated and purified. A single amino acid replacement was found, threonine for alanine at position 309 in the sequence (the numbering used is from Eu, a human γ1 myeloma IgG). This position is in the $C_{\gamma}2$ region of the heavy chain. In our studies of the Fc fragment, the amino acid sequence of the $C_{\gamma}2$ region of rabbit IgG has been completed and a high degree of homology (80%) of constant region domains of rabbit and human γ-chains became apparent. Perhaps this high degree of homology in the $C_{\gamma}2$ region reflects conservation of sequences required for complement fixations or other functions.

4. Mouse heavy chains

Mouse myeloma with antibody-like activity to phosphoryl choline have been described. They are of the γA type having kappa-type light chains. One exception is MOPC 511. Both immunologically and chemically this tumor has been shown to have both kappa and lambda light chains. In the urine the ratio is 1:9 but the reverse is true for the specific γA purified from an immunoabsorbent. This observation is very important in view of the possibility that a single clone of cells may produce three different variable regions. Studies are under way to obtain such evidence by the use of tissue culture cloning.

In the translocation hypothesis of the genetic control of immunoglobulins, gene translocation should prevent any single immunoglobulin-producing cell from making more than one type of light chain and one type of heavy chain. The finding of two light chains in a single clone associated with α-chains would introduce further constraints to the translocation hypothesis.

Serial No. NCI-3229

1. Laboratory of Cell Biology
2. Cell. & Tumor Imm. Section
3. Bethesda, Md.

The α-chain of MOPC 511 has been compared in its amino acid sequence of the first 15 amino acid residues with the α chains of 167 and 603; both of these tumors have antibody-like activity against phosphoryl choline. A single amino acid substitution has been found between the three heavy chains, demonstrating that a close similarity exists between these myelomas. Amino acid sequence is now continuing in order to define the hypervariable regions because they may be the areas of the immunoglobulin molecule involved in antibody specificity.

1. Laboratory of Cell Biology
2. Cell. & Tumor Imm. Section
3. Bethesda, Md.

Publications:

Strober, S., Appella, E., and Law, L. W.: Serological and immunogenic activity of soluble mouse transplantation antigens controlled by the H-2 locus. Proc. Nat. Acad. Sci. 67: 765-772, 1970.

Appella, E.: Amino acid sequences of two mouse immunoglobulin lambda chains. Proc. Nat. Acad. Sci. 68: 590-594, 1971.

Strober, S., Appella, E., and Law, L. W.: Characterization of the immunogenic and serological activity of soluble H-2 transplantation antigens in the mouse. Trans. Proc. (In press).

Appella, E., Chersi, A., Mage, R. G., and Dubiski, S.: The structural basis of the A14 and A15 allotypic specificities in rabbit immunoglobulin-G. Proc. Nat. Acad. Sci. (In press).

Serial No. NCI-5552
1. Laboratory of Cell Biology
2. Cell. & Tumor Immunol. Sect.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 26, 1970 through June 30, 1971

Project Title: Production and analysis of hybrid somatic cells: their application to some problems of importance in biology and medicine

Previous Serial Number: None

Principal Investigator: Hayden G. Coon, Ph.D.

Other Investigators:
Dr. George R. Martin (D:LB)
Dr. Peter C. Meuller (Post-doctoral Fellow, D:LB)
Dr. Marshall Nirenberg (H:LBG)
Dr. Sve Vogel (Post-doctoral Fellow, H:LBG)
Dr. Joseph A. DiPaolo (C:BGY-4629)
Dr. Igor B. Dawid (Not NIH)
Dr. John H. Sinclair (Not NIH)
Mr. Andrew Okot (Not NIH)
Mr. G. David Trisler (Guest Worker, C:LCB)

Cooperating Units: None

Man Years:
Total - 1 1/2
Professional - 1
Other - 1/2

Project Description:

1. Collagen synthesis in hybrid cells.

Collagen synthesis has been studied in cell strains produced by in vitro hybridization of fibroblasts (collagen synthesizing: col^+) with lymphocytes and plasmacytomas (non-collagen synthesizing: col^-). In collaboration with Dr. Lewis N. Lukens (Department of Biology, Wesleyan University, Conn.) Dr. George R. Martin and Dr. Peter Meuller (D:LB) we have found that hybrids between mouse fibroblasts and rat lymphocytes ($col^+ \times col^-$) do produce collagen, indicating that the negative control of the lymphocyte parent is not operating in these hybrids. Mouse fibroblasts hybridized with human lymphocytes also produce collagen. Homospecific crosses have been performed between a cloned line of mouse plasmacytoma (MOPC 315) which doesn't produce collagen and the same mouse fibroblast line employed with the lymphocytes, and these hybrids too, do produce collagen. Furthermore, the amount of collagen produced by all of the hybrids in each of these three cases has been found to be two to five times

greater than the original collagen producing mouse fibroblast. In the rat lymphocyte by mouse fibroblast hybrid, it was possible to detect alpha-2 chains specific to the rat by column chromatography. This result indicates that positive control or specific gene "turn on" has been detected in these hybrid cells.

Recently we have examined the collagen produced by heterokaryons formed by fusing chicken erythrocytes with human fibroblasts (the system in which Henry Harris has made the extraordinary observation that the erythrocyte nuclei become "reactivated" and reinitiate DNA, RNA and protein synthesis). Preliminary results indicate that after an initial reduction in collagen synthesis, it is resumed in heterokaryon cultures and chicken specific alpha-2 collagen polypeptide may appear. This result implies that nuclear fusion may not be a necessary prerequisite for gene "turn on". Because chick nuclei may be reisolated from heterokaryons, a number of new experiments can be undertaken.

2. Somatic fertilization: hybrid cells produced by fusion of spermatozoa or spermatids with fibroblast cells.

Germ cell by somatic cell hybrids interest us in three different applications. First, the genome of the germ cell might be supposed to be particularly susceptible to "turn on" of genetic functions when associated with specialized cells as heterokaryons or as hybrid cell strains. We have undertaken an examination of the "reactivation" of the sperm cell nucleus after fusion with fibroblast cell strains in the same way as Henry Harris has examined "reactivation" of chick erythrocyte nuclei after fusion. Secondly, the introduction of a haploid genome into a hybrid cell holds out the tantalizing possibility that haploid cell strains might be derived from the hybrid cells. Hybrids produced by fusion of a mouse spermatozoan with a human fibroblast might be expected to lose the human chromosomes selectively (as fusion between somatic cells do) which could leave a haploid mouse cell. Thirdly, sperm cell by somatic cell hybrids offer the possibility of deducing recombination frequency maps for the genes on individual human chromosomes. Since the selective loss of human chromosomes from human/mouse hybrids was described by Weissand Green in 1967, it has been apparent that a potentially powerful method for discovering linkage relationships of human genes was at hand simply from assays of the association of genetic functions in segregated hybrids from which many human chromosomes have been lost. This kind of test can give information only about linkage, i.e.,whether or not two functions are associated with the presence of the human chromosome. The other half of the formal genetic analysis, the distance between the genes and the linear order of genes on a given chromosome, may be found by analysis of the several functions (already known to be linked) in the sperm cells of a man who is known to be heterozygous for the traits studied. The different spermatozoa each represent recombination events in a haploid set of chromosomes. Unfortunately it is usually not possible to identify different

genetic properties in single spermatozoa, however, hybrid cell strains made between sperm and fibroblast cells might be grown to sufficient numbers to permit assay of the individual activated gene products. An analysis of the frequency with which linked markers are present in different, independently isolated sperm/fibroblast hybrids should give a number which is proportional to the distance separating the markers (the recombination frequency map of the chromosome).

In collaboration with Dr. Martin Rechsteiner (Department of Biology, The University of Utah) and Mr. Andrew Okot (Department of Zoology, Indiana University) we have been trying to produce hybrids between fibroblasts and spermatozoa or spermatids. Both ventures have now succeeded. Rechsteiner in Utah and we have independently produced hybrids between human spermatozoa and mouse fibroblasts. Hybrids between hamster and rat sperm and mouse fibroblasts are also being studied. Okot has obtained hybrids between rat spermatids and mouse fibroblasts. We are all cooperating in the analysis of this most important new class of hybrid cells.

The production of a haploid human cell strain would depend upon the selective loss of the chromosomes of another species from a human/x hybrid. Human chromosomes are selectively lost, however, from all of the hybrid cell combinations described thus far. Our laboratory found that human chromosomes remain and chicken chromosomes are lost from human-chicken hybrid cells. The segragation of chicken chromosomes is rapid and complete. If hybrids can be produced between human spermatozoa and chicken cells then it is quite possible that haploid human cell lines will be developed.

3. The fate of mitochondrial DNA in human/mouse and hamster/mouse hybrid cells.

The chromosomes are the only cell organelles which have been traced during the evolution of lines of hybrid cells propagating in vitro. In collaboration with Dr. Igor B. Dawid (Carnegie Institution of Washington, Department of Embryology, Baltimore) and Dr. John Sinclair (Department of Zoology, Indiana University) we are attempting to follow the mitochondrial DNA in human/mouse hybrid cells. Dawid has found that (exclusive of the genes for ribosomal RNA) there is very little molecular hybridization (DNA-DNA or DNA-RNA) between the mitochondrial DNAs (m-DNA) of two closely related species of the genus Xenopus (frogs), and very little or no hybridization of any mitochondrial DNA with the nuclear DNA of the same species. Purified mitochondrial DNA from mouse and human cell cultures will be used as template to produce complimentary ^{32}P-RNA to mouse m-DNA and ^{3}H-RNA to human m-DNA respectively. The two reagents may then be used singly or in combination in standard Millipore filter DNA-RNA hybridizations to determine the amount of mitochondrial DNA typical of each species found in the parents and the human/mouse hybrids. Since there is no cross-hybridization with nuclear DNA very little purification of hybrid cell DNA is required in order to do the tests. A particular advantage of this technique is that it can be used with the small number of cells appropriate to the detection of

m-DNA in the cell cultures of relatively new hybrids from which few of the human chromosomes have been lost by segregation.

4. Isolation of individual human chromosomes from segregated human-mouse hybrid cells.

The resolution of studies of the synthetic capacities of "chromatin" and the comparison of the molecular hybridization of DNA and RNA from different tissues would be greatly increased if individual chromosomes could be isolated separately from one and other (and in sufficient quantity for biochemical analysis). Attempts to separate metaphase chromosomes according to their behavior in gradients have proven somewhat disappointing. While theoretically it should be possible to achieve separation by gradient centrifugation techniques, the best results reported by Salzman et al., (I:LBV) have been separations based on size of large ones from small ones. A new approach to this question is suggested by the fact that individual human chromosomes (especially chromosome #17, containing the thymidine-kinase gene) may be "trapped" alone in human/mouse or human'hamster hybrids under selective conditions in which the life of the cell depends on the presence of a particular human gene. Hybrids which have lost all of the other human chromosomes by segregation during extended replication of the hybrid cells, as originally described by Weiss and Green in 1967, may contain several copies of the single required human chromosome. Starting with such a hybrid cell which contains the mouse "L" cell genome plus the single human chromosome, the problem of obtaining preparations of that single human chromosome becomes a new one: separation of a human chromosome from a group of mouse chromosomes rather than from a group of human chromosomes. Two approaches which take advantage of species differences are being investigated. If antibodies can be raised specific to the liberated mouse metaphase chromosomes (and which do not cross react with the human chromosomes), then it is possible that immune-adsorbant columns would permit chromosome separations directly. Alternatively, it can be calculated that the forces involved in DNA-DNA or DNA-RNA molecular hybridization of the mouse satellite DNA should be sufficient to retain chromosomes of the mouse cellulose column with mouse satellite DNA. Either the antibody method or the satellite hybridization method might possibly be adapted to achieve selective increase in the density of the mouse chromosomes sufficient to permit absolute separation by differential centrifugation.

5. Malignancy in hybrid cells between malignant and non-malignant cell strains and between malignant cells of different origin.

In collaboration with Dr. Joseph DiPaolo (C:BGY-4629) we have produced hybrid cells between a malignant hamster cell line (OBP) induced by benzpyrene treatment and a thymidine-kinase deficient derivative of the well known BHK strain. These cells will be tested for evidence of malignancy

in young, irradiated hamsters. A correlation will be sought between the time at which malignant growths appear and the chromosomal constitution of the hybrid strains used. This study has been undertaken in a preliminary way to test the feasibility of developing a larger program here at NIH like that being conducted by Harris and Klein. It is anticipated that our program if undertaken, will have a slightly different emphasis; we are interested in searching for evidence of complementation groups among different malignant cells by cellular hybridization.

6. Culture of differentiated cells for use as parents in cellular hybridizations.

It has been a long standing interest of this laboratory to investigate the specialized characteristics of differentiated cells in culture. Recently, in the course of an attempt to culture normal rat neurons or neuroblasts from retina and brain of early embryos (undertaken in collaboration with Dr. Nirenberg and Dr. Sve Vogel (H:LBG), our laboratory has found that ciliated ependymal cells may be propagated in clonal cell culture. The existence of strains of ciliated mammalian cells may prove to have wide application, not only as a marker in cell fusions but also with respect to in vitro tests of the physiology of ciliary action, and the isolation of basal bodies. Because of the probable ependymal origin of these particular ciliated cell strains, the special transport physiology as well as the susceptibility to pathological agents of this strategic cell type may be studied in vitro for the first time.

Serial No. NCI-5551

1. Laboratory of Cell Biology
2. Cell. & Tumor Immunol. Sect.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of in vitro tests to detect tumor-specific transplantation antigens. Several techniques are currently under development

Previous Serial Number: NCI-5551

Principal Investigator: Peter W. Wright, M.D.

Other Investigators: Dr. L.W. Law (C:LCB-3200)
Dr. E.A. Appella (C:LCB-3Z29)

Man Years: Total - 2
Professional - 1
Others - 1

Project Description

(1) A cytotoxic assay using ^{51}Cr-labelled target cells has been employed to detect a tumor-specific surface antigen present on a variety of SV40--transformed mouse and hamster cell lines. Cytotoxic inhibition can be used as a quantitative measure of surface antigen concentration and specific activity. The cytotoxic assay overcomes many of the disadvantages of currently available techniques used to detect tumor-specific antigens: it is simple, sparing of material, rapid, objective, reproducible and quantitative. It is hoped that the cytotoxic assay will allow rapid progress in further biological and biochemical characterization of the SV40 tumor-specific transplantation antigen (collaborative work with Drs. Appella and Law).

(2) A microcytotoxicity assay, modified from the colony inhibition test initially devised by the Hellström's has recently been developed in this laboratory. This technique has been used to detect a cell-mediated immune response to both histocompatibility and tumor-specific transplantation antigens. A protective or "blocking" effect of immune serum can be demonstrated. This technique will be utilized to clarify the nature of this "blocking" phenomenon.

(3) A macrophage migration inhibition assay has also been developed. This technique also provides a measure of cell-mediated immunity, and will be utilized to complement the microcytotoxicity assay described above. In studies accomplished to date in collaboration with Dr. I. Bernstein (C:ET-4665) this test appears to be capable of detecting enhancing antibody.

Serial No. NCI-5551

1. Laboratory of Cell Biology
2. Cell & Tumor Immunol. Sect.
3. Bethesda, Md.

Publications:

Bernstein, I. and Wright, P.W. In vitro defection of enhancing antibody by the macrophage migration test. Nature (In press).

Wright, P.W. and Law, L.W. Quantitative in vitro measurement of SV40 tumor-specific transplantation antigens (TSTA's). Proc. Nat. Acad. Sci. (In press).

Serial No. NCI-5556
Formerly NCI-3202
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathogenesis of plasma cell neoplasia in mice; studies on antigen-binding myeloma proteins; genetic basis of antibody (immunoglobulin) diversity; screening of human myeloma proteins for antigen-binding activity

Previous Serial Number: NCI-3202

Principal Investigator: Michael Potter, M.D.

Other Investigators:
Mr. David McKean (Graduate Student, Johns Hopkins University)
Dr. S. Rudikoff (Staff Fellow, NCI)
Dr. R. Asofsky (I:LGAR-116c)
Miss R. Lieberman (I:LCI-15c)
Dr. L. Hood (California Institute of Technology)
Dr. W. Konigsberg (Yale University)
Dr. K. Sibinovic (Bionetics Research Laboratories)

Cooperating Units: None

Man Years:
Total - 4
Professional - 1
Others - 3

Project Description:

I. Pathogenesis of Plasma Cell Tumor Development.

The inbred BALB/c strain of mice is highly susceptible to plasma cell tumor development. Plasma cell tumors can be induced in 60-80% of mice following intraperitoneal injection of mineral oils or chemically defined isoparaffins (e.g. Pristane). The protocol for tumor induction involves giving a total of three intraperitoneal injections, each of 0.5 ml, when the mice are 2, 4 and 6 months of age. The latent period is between the second and sixth month of age. Three factors appear to be involved in the pathogenesis of plasma cell tumors; 1) the specific genetic constitution of the BALB/c mouse (other strains excepting NZB are resistant), 2) intraperitoneal granuloma - a tissue which specifically favors neoplastic transformation of plasma cells and 3) natural antigenic stimulation (germfree BALB/c mice which lack a gastrointestinal microbial flora develop a very low incidence of plasma cell tumors following mineral oil injection). Roughly 60% of all plasma cell tumors produce IgA type myeloma protein suggesting the IgA system of immunocytes is selectively involved in plasma cell tumor development.

Serial No. NCI-5556
Formerly NCI-3202
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

A. Genetic Factors

The specific genes and their respective products in the BALB/c mouse which makes this strain susceptible to plasma cell tumor development are not known. We are currently exploring several experimental approaches in attempts to find or identify characteristics in the BALB/c mouse which are related to plasma cell tumor development. First, in the last year through the kindness of Dr. Don Bailey of the Jackson Laboratory, Bar Harbor, Maine, we have obtained seven new inbred strains of mice that were derived from a cross between BALB/c (plasma cell tumor susceptible) and C57BL (plasma cell tumor resistant). We are breeding all seven of these strains and are testing them for plasma cell tumor susceptibility. The rational for this approach is that if a few genes are involved in susceptibility, these may by chance have been segregated in one of the seven strains.

It has recently been observed that mice differ in their ability to produce high levels of circulating antibody. Two strains of mice have recently been developed by Biozzi in Paris, one which develops high levels of circulating antibody and the other low levels. These characteristics are heritable and can be maintained by restrictive breeding of these stocks. Possibly immunoglobulin level variations are a function of the degree of plasma cell proliferation following antigenic stimulation. Accordingly, we are investigating the serum levels of IgA and IgF classes following the injection of mineral oil into BALB/c and C57BL mice to determine if there is an exaggerated response in BALB/c. The working hypothesis is that if the neoplastic transformation is a chance event, one would be more likely to observe these changes when the precursor populations have been expanded. Early indications are that IgA levels are low in BALB/c mice, even during the latent period before plasma cell tumors appear. However, at the time the tumors begin to appear these levels also begin to increase. It is essential then to study in detail how and when the levels increase. Further, comparative studies in C57BL are underway and also in the seven congenic strains listed above.

The recent studies on Xeroderma pigmentosa in man have suggested that a genetic defect affecting the repair of DNA may be involved. Presumably, the defective DNA repair mechanism permits the accumulation of somatic mutations, some of which affect growth regulation. Possibly, the BALB/c mouse also has a defective DNA repair process and hence accumulates somatic mutations in plasma cells. If so, it is hypothesized that agents which would increase the rate of somatic mutation might have a co-carcinogenic effect in plasma cell tumor development. We have in progress experiments on plasma cell tumor induction in which BALB/c mouse were treated during the first four months (latent period) with whole body X-irradiation, or ethyl methane sulfonate, or M-methyl nitroso-urea. In the first experiment with these agents, the co-carcinogenic materials were given in the middle of the latent period. As

more is learned about the serum IgA levels during the mineral oil treatment phase, it may be essential to repeat the protocol but giving the co-carcinogenic materials near the end of the latent period.

As age is a major factor in a development of human myeloma and benign monoclonal gammopathy, we have underway an experiment comparing the ability of 12 and 13 months old BALB/c mice to develop plasma cell tumors following mineral oil induction. These mice have now gone through the latent period and are beginning to develop plasma cell tumors. The rational in these experiments is that the stem cells or memory cells which are precursors of plasma cell tumors may accumulate somatic mutations during life. The older the stem cell the more likely the chance for "mutational" events to have occurred.

Intracisternal type A particles have been found in all induced plasmacytomas. Viruses, however, have not been implicated directly as the cause of plasmacytomas. Most of these experiments are based on the inability to induce plasmacytomas in BALB/c mice with extracts of plasmacytomas enriched with the intracisternal type A virus particles. Viruses, however, with a predilection for plasma cells could hypothetically bring about neoplastic transformation. In view of recent findings that BALB/c mice and also some transplantable plasmacytomas contain the GS antigen (murine leukemia virus antigen) the possibility was considered that the intracisternal type A particle might in some way be related to this complex of agents. Accordingly, in collaboration with Dr. Lloyd W. Law (NCI-5553) BALB/c mice have been injected with mineral oil and during the latent period infected with Moloney leukemia virus to determine if the incidence of tumors is accelerated.

B. Antigenic Factors

Following the observation of McIntire and Princler that germfree BALB/c mice develop a reduced incidence of plasma cell tumors presumably due to an absent microbial flora, we have attempted further experiments to determine if monocontamination would restore plasma cell tumor susceptibility. In collaboration with Dr. Richard Asofsky (I:LGAR-116c), we have found that BALB/c mice monocontaminated with Salmonella tel aviv survive moderately well under germfree conditions. The Salmonella tel aviv strain was chosen because it had been previously observed that 2 myeloma proteins specifically precipitated with the O-specific antigen produced by this species of Salmonella. Despite the fact that a number of the mice monocontaminated with Salmonella tel aviv developed unusual illnesses months after the initiation of the monocontamination which unfortunately resulted in the loss of many of these animals before autopsies could be done, we nonetheless were able to observe a slightly increased incidence of plasma cell tumors. During the course of these experiments, however, it became apparent from other publications and our experiences with monocontamination that monocontamination such as we had

initially performed was not the best means for studying antigenic stimulation in germfree BALB/c mice. It was considered that antigen feeding would be more effective. Heremans and colleagues in Belgium have shown that feeding of antigens to germfree mice results in a predominantly IgA type immune response. We are currently initiating an experiment in germfree mice in which various antigens will be fed during the latent period. One of the principal aims of this work is to find the method for inducing tumors that produce antigen-binding myeloma proteins to the antigen used to stimulate the germfree mouse. This is deemed important because it will permit us to trace the natural history of the neoplastic plasma cell from its normal progenitor.

We have extended our study of the normal microbial flora of BALB/c mice via the open-ended contract at Bionetics Research Laboratories and also with some facilities on NIH-69-2142 contract with BRL. Dr. K. Sibinovic has been made principal investigator on our NIH-69-2142 contract and she has available a diagnostic bacteriological laboratory. We have submitted to her numerous specimens from BALB/c mice in our colony and she has isolated from these materials approximately 40 different organisms. Antigenic materials have been extracted from these organisms. The antigens have been used to screen myeloma proteins for antigen-binding activity. We have found that many of the organisms which are potential pathogens and which also were in frequent isolates, for example, Herellea vaginicola, Mima polymorpha, Proteus morganii, and various Pasteurella species produce antigens which are bound by myeloma proteins in our collection. This result suggests that potential pathogens may be capable of invading the gut wall from time to time and stimulating IgA producing cells. We have been unable, however, to directly trace the IgA producing plasmacytomas to a normal precursor population.

As a by-product of our experience with screening myeloma proteins in mice to bacterial antigens, we have begun to establish a minimal screen for human myeloma proteins. Again in collaboration with Dr. K. Sibinovic of BRL, Litton Industries, we have prepared antigens from a number of bacterial species. We anticipate an initial screening program to involve a basic screen of approximately 50 antigens and anticipate in the future of increasing this to approximately 100. The human myeloma proteins are being tested for their ability to precipitate with polyvalent antigens from microbial plant and other sources. These antigens are primarily polysaccharide antigens and hence are ideal for testing for precipitating activity. A series of 187 human myeloma sera have been given to us by Dr. Elliott Osserman (Francis Delafield Hospital, New York) for this purpose and also Dr. William Terry (NCI) has given us samples of myeloma proteins made available to him. We hope by the end of this year to have an ongoing screening process which could be made available to any clinical laboratory in the U. S. or elsewhere who wished to have a myeloma protein screened for activity. The basic problems of course in establishing such a service are first to assemble the critical antigens and to be assured that they are active. The second problem is to

obtain sera and to properly store these sera during the testing procedure so that the proteins will not be denatured by repeated freezing and thawing. In an initial survey of Dr. Osserman's lab 187 sera 2 sera were discovered that precipitated with Hemophilus influenzae type B polysaccharide (polyribitol phosphate) which was kindly supplied to us by Dr. John Robbins (NICHD).

C. Oil Granuloma

The peritoneal oil granuloma factor in plasma cell tumor development is poorly defined. It appears to be a permissive tissue site for neoplastic transformation. Plasma cells elsewhere in the organism, e.g. the lymph nodes apparently do not undergo neoplastic transformation in BALB/c mice. The oil granuloma may be a selective site for colonization of IgA producing cells and factors which promote colonization may be important. Little is known, however, about this process. Hollander and colleagues in New York have made the interesting observation that testosterone excellerates plasma cell tumor development in BALB/c mice. We are currently repeating this work and will attempt to determine if testosterone increases disproportionately the IgA level in oil injected mice. The process of colonization or plasma cell hyperplasia in the oil granuloma will be evaluated by the histologic methods.

II. Studies on Antigen-Binding Myeloma Proteins

The screening of myeloma proteins in mice for antigen-binding activity continues to be productive of new types of activity. A large basic screen has been developed which contains approximately 60 antigens. These antigens include all those which have previously shown to be active, antigens from the normal microbial flora of the BALB/c mice, and a variety of other available antigens of potential interest. Myeloma proteins that bind arabinogalactan (Larch wood hemicellulose), a common trypsin-sensitive antigen in Salmonella, Hemophilus influenzae type B polysaccharide (a polyribitol phosphate) and antigens from several bacterial species (see above) have been recently identified. Studies are designed to establish the incidence of antigen-binding activities in a consecutive series of myeloma proteins. Further, these studies are being extended in order to find multiple examples of myeloma proteins which bind this same antigen. To accomplish this experimental aim, a consecutive series of tumors must be available and these also must be preserved in a frozen tumor bank so that when an active protein is found the source of this protein can be recovered. One of the immunochemical products of such a search is to find a myeloma protein that could be crystallized and also have antigen-binding activity to a chemically characterized determinant.

Specific studies on the primary structure of anti-phosphoryl choline binding IgA myeloma proteins are in progress. These involve collaborations with Dr. L. Hood (Cal Tech) and Dr. W. Konigsberg (Yale University) to determine

if 2 independently induced IgA myeloma proteins, HOPC 8 and TEPC 15, make an identical protein. Large quantities of these proteins purified by immunoabsorption are being supplied to these workers.

In addition, comparative primary structural studies on MOPC 167, MOPC 511 and McPC 603 (all IgA anti-phosphoryl choline binding proteins) are being done at the NCI. This work is being done by Dr. Stuart Rudikoff (Staff Fellow, NCI) who is purifying the IgA myeloma proteins on immunoabsorbant columns and developing methods for getting quantitative isolation of the polypeptide chains. He is working with Dr. Ettore Appella (NCI-5551) on the primary structure problem. The chief emphasis on this work is to compare the two proteins, MOPC 167 and MOPC 511, which specifically bind choline-containing antigens (and do not require the presence of the phosphoryl group).

The studies of the primary structure of the phosphoryl choline binding group of myeloma proteins is of particular interest since Dr. Henry Metzger (A:A&R-8c) and Dr. Bruce Chesebro (A:A&R) with whom we have close working arrangements have recently developed methods for affinity site labelling the anti-phosphoryl choline binding myeloma proteins.

III. Genetic Basis of Antibody Specificity.

In the last several years, in collaboration with Miss Rose Lieberman (I:LCI-15c) we have produced myeloma-specific antisera by immunization of other strains of mice with BALB/c myeloma proteins of the IgA and IgG classes. A myeloma-specific antiserum is usually specific for the immunizing antigen alone and will not cross react with any other myeloma protein. The use of myeloma-specific antisera provides a powerful tool for estimating the total number of specific types of immunoglobulins in the BALB/c mice. We are currently attempting to screen a 100 myeloma proteins with 25 different myeloma-specific antisera in a search for repeats. We have been successful in finding repeats of the anti-phosphoryl choline myeloma proteins. We are continuing to make new antisera to the myeloma proteins that have specific binding activity in hopes of elucidating new groups of similar proteins. We are also hoping to develop methods for finding immunoglobulin molecules in normal antibody that share individual antigenic specificities with myeloma proteins and in particular with myeloma proteins that have known antigen-binding activity.

In the last year in collaboration studies with Dr. Lee Hood (Cal Tech) and Mr. David McKean (Graduate Student, Johns Hopkins University), we have shown that the kappa chains in BALB/c mice have a remarkable degree of variability within the first 23 residues from the amino terminus. At the present time, over 18 subgroups have been delineated among approximately 30 chains so far examined. Several of these sequences were also determined by Dr. Ettore Appella (NCI-5551). This unique property of BALB/c kappa chains

not found in the kappa chains of any other species so far, permits subgrouping kappa chains from a readily accessible peptide, mainly the amino-terminal Cys 23 peptide. All unblocked BALB/c kappa chains can be characterized by amino acid sequence determination on the Beckman Amino Acid Sequencer. Currently, in collaboration with Mr. David McKean, we have gone on to explore in detail a group of 4 proteins which possess the same amino-terminal Cys 23 peptide, e.g. MOPC 70, MOPC 321, MOPC 63 and TEPC 124. Mr. McKean has developed methods for isolating the peptides of this group of kappa chains and has established a tentative primary structure for the MOPC 321 protein. He has found a considerable degree of variation between the MOPC 70 and MOPC 321 sequences. Further sequence analysis is designed to find proteins which show less variations between each other in the hopes of finding some explanation for the origin of variation. Currently this work is proceeding well.

We are continuing to produce new strains of mice which are co-isogenic derivatives of BALB/c that contain immunoglobulin heavy chain linkage groups (C_H) from other sources. These include: BALB/c·C57BL IgC_H (formerly called BALB/c 2/2); BALB/c·AL IgC_H; BALB/c·Kyushu IgC_H and BALB/c·DBA/2C_H. A large series of plasma cell tumors is being induced in BALB/c·C57BL IgC_H. The myeloma proteins from these tumors are a source for finding genetic variations in the V_H polypeptides.

Serial No. NCI-5556
Formerly NCI-3202
1. Laboratory of Cell Biology
2. National Cancer Institute
3. Bethesda, Md.

Publications:

Finlayson, J. S., Mushinski, J. F., Hudson, D. M., and Potter, M.: Components of the major urinary protein complex of inbred mice: Separation and peptide mapping. Biochem. Genetics 2: 127-140, 1968.

Anderson, P. N., and Potter, M.: Induction of plasma cell tumors in BALB/c mice with 2,6,10,14-tetramethylpentadecane (Pristane). Nature 222: 994-995, 1969.

Metzger, H., Potter, M., and Terry, W.: Summary of workshop on homogeneous immunoglobulins with binding activity. Immunochemistry 6: 831-836, 1969.

Lieberman, R., and Potter, M.: Crossing over between genes in the immunoglobulin heavy chain linkage group of the mouse. J. Exp. Med. 130: 519-541, 1969.

Jaffe, B. M., Eisen, H. N., Simms, E. S., and Potter, M.: Myeloma proteins with anti-hapten antibody activity: ε-2,4-dinitrophenyl lysine binding by the protein produced by mouse plasmacytoma MOPC 460. J. Immunol. 103: 872-874, 1969.

Potter, M.: Mouse IgA myeloma proteins that bind polysaccharide antigens of enterobacterial origin. Fed. Proc. 29: 85-91, 1970.

Potter, M.: Myeloma proteins with antibody-like activity in mice. Miami Winter Symp. Amsterdam, The Netherlands., North-Holland Publishing Co., 1970, 2: 397-408.

Smith, A. M., Wivel, N. A., and Potter, M.: Plasmacytopoiesis in the pronephros of the carp (Cyprinus carpio). Anat. Rec. 167: 351-370, 1970.

Potter, M., and Lieberman, R.: Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphoryl choline. J. Exp. Med. 132: 737-751, 1970.

Hood, L. E., Potter, M., and McKean, D. J.: Immunoglobulin structure: Comparison of amino terminal sequences of kappa chains derived from genetically similar mice (BALB/c). Science 170: 1207-1210, 1970.

Potter, M.: Myeloma proteins (M-components) with antibody-like activity. New England J. Med. April, 1971, in press.

Serial No. NCI-5554
Formerly NCI #3209

1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A. Pathogenesis and pathophysiology of plasma cell neoplasms in mice and characterization of their immunoglobulin products
B. Characterization and measurement of soluble antigens associated with human neoplasms

Previous Serial No: NCI-3209

Principal Investigator: K. Robert McIntire, M.D.

Other Investigators: Dr. Philippe P. Sizaret, I.A.R.C., Lyon, France
Dr. Cedric Bryan, University of Missouri School of Medicine, Columbia, Mo.
Dr. Lloyd Old, Sloan-Kettering, New York
Dr. Charles Vogel, Uganda Cancer Institute, Kampala, Uganda

Cooperating Units: I A R C, Lyon, France
Uganda Cancer Institute, Kampala, Uganda

Man Years: Total - 2
Professional - 1
Other - 1

Project Description:

1. Plasma cell carcinogenesis

The results of combining cell free extracts of plasma cell tumors with mineral oil during the induction of plasma cell tumors (PCT) are now complete. There was no increase in the incidence in either Germfree (GF) mice, where the expected incidence is low (4%) or in ex-GF mice where the incidence is high (77%) with mineral oil alone. Mice treated with tumor extracts and no mineral oil developed no PCT. There did appear to be a significantly longer latent period for PCT in GF mice in addition to the decreased incidence. The other part of the experiment was to determine an effect on the immunoglobulin product of the PCT arising in mice receiving PCT extract. The extracts were all from γM producing tumors; 1/2 from κ

type, 1/2 from λ type. There was no change in the expected incidence of λ proteins, but the γM producing tumors rose from 3% to 16% and there was a decrease in γA tumors from the expected 54% to 21%. In addition to the increase in γM PCT there was an increase in the 7S γ (all 3 classes considered together) from 23% to 37%. The interpretation of these findings is only speculative, but may be evidence for extra-chromosomal (episome) information directing the synthesis of immunoglobulins.

Hybrid (BALB/c X NZB/B1) F_1 mice have been treated with Pristane (2, 6, 10, 14--Tetramethylpentadecane) IP to induce PCT. Over half of the autopsied mice have had PCT, but the hoped for increase in lambda type tumors, which are very rare in BALB/c, has not materialized. This experiment is still in progress.

2. Studies on mouse macroblobulin (γM)

A second mouse γM myeloma ($M^{c}PC$ 1748) with antibody activity was discovered. This one has weak precipitating activity against the ligand DNP. The macroglobulin has been purified and is still active against DNP. Studies on the binding activity of whole molecule and its sub-units are in progress.

3. Studies on mouse light (L) chains

Technical difficulties in quantitation of mouse immunoglobulin L chains are still being worked on. A method for separation of heavy (H) and L chains after partial reduction utilizing DEAE cellulose equilibrated in 7M urea can be accomplished in 1 hr. as compared to a 48-72 hr. procedure when Sephadex is used.

4. Myeloma kidney in mice with PCT

The effect of diuresis and antidiuresis on the formation of tubular casts in mice with a transplantable Bence Jones (BJ) protein-producing PCT was studied with Dr. C. Bryan. Mice treated with 0.5% furosemide in their diet drank 6.4 ml water/day--while those given limited access to water drank 0.9 ml/day; normal intake of mice given water ad lib was 2.7 ml/day. The degree of cast formation/tumor weight was greatest in the dehydrated mice (39%/gm) and least in the mice with chronic diuresis (13%/gm); the controls were intermediate (25%/gm). These differences were significant and demonstrated that chronic diuresis retards the rate of cast deposition in mice with BJ proteinuria.

5. Tumor surface antigens

A direct cytototoxicity assay testing the effect of immunoglobulin class specific antiserum on PCT's indicates that cells secreting γF have immuno-

globulin receptors at the cell surface. Thus far Fc determinants have been located on one PCT line and F ab determinants on another. In general, non-γF PCT are not affected by the anti-γF antiserum and other class specific antiserums do not affect the γF PCT.

In collaboration with Dr. Lloyd Old (Sloan-Kettering Institute for Cancer Research, New York) various populations of mouse lymphoid cells have been tested with the class-specific antiserums. Only kappa and γM receptors have been demonstrated and these in a characteristic per cent for bone marrow, lymph node and spleen. Thymocytes were negative for immunoglobulin determinants.

6. Alpha-Fetoprotein (αFP) quantitation

With Dr. P. Sizaret, visiting scientist from I.A.R.C., Lyon, France a technique for the quantitative measurement of αFP has been devised using the method of electroimmunodiffusion. Routinely the technique will measure down to 350 nanograms. With a modification using radioactive antiserum and autoradiography, levels as low as 20 nanograms αFP can be measured.

The technique is being used to quantitate serum levels of patients with hepatoma from Uganda in collaboration with Dr. C. Vogel (Uganda Cancer Institute). The quantitation is also being applied to monkey serums where the monkeys are undergoing treatment for hepatocellular carcinogenesis. Results at this time indicate a very wide range of serum αFP for different hepatomas.

7. Pulmonary tumor antigen

Studies have been undertaken with Dr. Sizaret to define a soluble antigen of primary pulmonary tumors similar to the carcinoembryonic antigen described by P. Gold. Saline extracts of pulmonary tumors have been used to immunize rabbits. An antigen common to 5 of 10 primary lung tumors can be differentiated from all antigens of the normal lung, but unfortunately the antigen, or one of cross-reacting specificity, is found in normal tracheal and esophageal extracts. This work will be continued with more primary lung tumors.

Serial No. NCI- 3222

1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genetic and biochemical mechanisms controlling protein synthesis in well-differentiated neoplastic cells, mouse plasma cell tumors

Previous Serial Number: NCI-3222

Principal Investigator: J. Frederic Mushinski, M.D.

Other Investigators: Dr. M. Potter (NCI-5556)
Dr. P. Periman (NCI-3244)
Miss R. Lieberman (I:LCI-15c)
Dr. Gunter von Ehrenstein, Max-Planck Institute for Experimental Medicine, Göttingen, Germany
Dr. Harold P. Morris, Howard University

Cooperating Units: Max-Planck Institute for Experimental Medicine, Göttingen, Germany
Howard University

Man Years: Professional - 1
Other - 1/4

Project Description:

A. Mouse IgA Myeloma Proteins

Work has progressed in the comparison of the two types of mouse IgA immunoglobulins as exemplified by the products of two sister-line plasma cell tumors, Adj.PC 6A and Adj.PC 6C. These two tumors were isolated as individual peritoneal granulomatous nodules from a single BALB/c mouse which had received injections of adjuvant. The tumors developing from these nodules have been transplanted separately for more than one hundred transplant generations over a period of nearly ten years. Both tumors secrete IgA myeloma proteins, but only the product of Adj.PC 6A (6A protein) exhibits the properties commonly isolated with the IgA immunoglobulins. It is found in the blood serum, and it exists in several polymeric forms of a basic four-chain 7S subunit linked together by disulfide bonds. 6C protein, on the other hand, is found in the urine as well as in the serum, exists as a two chain, 3.9S unit which does not polymerize further. The one light and one heavy chain of 6C protein are covalently linked by disulfide bridges whereas the two light chains of 6A protein are disulfide linked to each other but not to the two heavy chains.

We have demonstrated that the heavy chains of these two proteins differ significantly from one another antigenically and chemically. It appears that the differences are located at the carboxyl-terminal portion of the heavy chains since we can demonstrate antigenic similarities between the Fab fragment (amino-terminal portion) of 6A protein and the intact 6C protein using homologous antiserums which, when absorbed, are idiotypically specific for the 6A and 6C proteins among all the IgA proteins tested. Other antiserums show antigenic groups located on the Fc fragment (the carboxyl-terminal portion) of 6A protein but not found on 6C protein. Five tryptic peptides have been found in the 6A Fc fragment, and in the Fc fragments of all of the polymeric-type mouse IgA immunoglobulins studied, which are absent in the tryptic peptides of 6C proteins and of all two-chain IgA molecules studied by two-dimensional mapping. Carboxypeptidase digestions have shown the carboxyl-terminal amino acid of 6A protein to be Tyrosine, while that of 6C protein is either Glutamine or Serine, but certainly not Tyrosine. Work is currently in progress to precisely determine the amino acid sequence of the carboxyl terminus of both heavy chains. In addition amino acid compositions have been done on all the tryptic peptides isolated from two separate digestions after separation of the peptides by paper electrophoresis and chromatography. Analysis is under way to try to locate the peptides shared by the light chains and the heavy chains of these two proteins and those unique to 6A or 6C. We will attempt to localize the carboxyl-terminal peptides from these four chains for sequence analysis. We hope that elucidation of the exact difference between these two IgA heavy chains will tell us how heavy chains are synthesized and what genetic lesion in this synthesis is responsible for production of the IgA half molecules.

Award: The William O. Moseley, Jr. Travelling Fellowship from Harvard University enabled continuation of this project at the Department of Molecular Biology, Max-Planck Institute for Experimental Medicine in Göttingen, Germany from October 1, 1969 through September 30, 1970.

B. Mouse plasma cell tumor transfer RNA

We have been extending our previous studies on the differences of aminoacyl-tRNA among different plasma cell tumors. Initially we demonstrated differences in Leu-tRNA's among plasma cell tumors which secreted only kappa-type Bence Jones proteins. We have subsequently examined the Leu-tRNA's, Ser-tRNA's and Tyr-RNA's from six myelomas which secrete complete IgA immunoglobulins, most of which have antibody activity to one or more haptens. These biologically similar tumors also demonstrated remarkable differences in aminoacyl-tRNA's when examined by reverse phase chromatography. The chromatography patterns of normal mouse organs were also examined in this way, and some reproducible differences were seen among these organs. The stability of these chromatographic patterns was demonstrated by comparing the patterns of tRNA from several different transplant generations of the same tumor. Additional

strength was afforded the argument that these aminoacyl-tRNA patterns were stable, determined characteristics of each individual tumor when the patterns for the two sister-line tumor discussed above, Adj. PC 6A and Adj. PC 6C were found to be identical. We have suggested that the pattern of tRNA's expressed in a cell or a clone of cells may be determined by the requirements of the particular messengers present in that cell. Myelomas might, then, differ one from another more than one normal organ differs from another, because the preponderant messenger in each plasma cell tumor has one unique structure as opposed to a large, varied population of messengers in each normal cell, especially those not especially differentiated toward secretion of a particular protein.

C. Rat hepatoma transfer RNA

To further pursue the biological significance of the above findings, we have begun to repeat several aspects of the aminoacyl-tRNA comparisons using a different animal species and a tumor of different histological type. We have prepared tRNA from three well-described carcinogen-induced Morris rat hepatomas. The hepatomas studies included one "minimal deviation" type, 7800, one rapid-growing type, 7777, and one intermediate between these two, 5123. Initial studies suggest that the aminoacyl tRNA's from these tumor differ in their chromatographic patterns more than the normal mouse organs described above but not to the extent seen upon comparing several plasma cell tumors. The effects of growth abnormalities is being studied by comparing the patterns of tRNA's from normal rat liver, from starved livers, and from regenerating livers. Initial results indicate very minor effects upon the chromatographic patterns from these physiological stresses, but a more thorough investigation into the tRNA patterns at a variety of times after partial hepatectomy is planned. Many other factors involved during extraction, preparation, charging, and storage will be studied for their effects on tRNA chromatographic patterns.

Serial No. NCI-3222

1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

Publication:

Mushinski, J. F. γA Half Molecules: Defective Heavy Chain Mutants in Mouse Myeloma Proteins, J. Immunol. 106: 41-50, 1971.

Serial No. NCI-5523
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Spontaneous association of enzyme subunits.

Previous Serial Number: Same

Principal Investigator: Sidney Shifrin

Other Investigators: Samuel W. Luborsky

Cooperating Units: None

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: Both β-galactosidase and L-asparaginase were studied. These enzymes from Escherichia coli B consists of four subunits which are held together by noncovalent bonds. Enzymatic activity of asparaginase is associated with the tetramer and the subunit is catalytically inactive. The purpose of this study is to examine the nature of the forces that are involved in the association process by following physico-chemical properties upon treatment with various dissociating agents such as urea, guanidinium chloride, sodium dodecyl sulfate and acid and alkali.

Methods Employed: The physico-chemical properties examined in this study were ultracentrifugation that included sedimentation velocity and sedimentation equilibrium, fluorescence and absorption spectroscopy as well as polyacrylamide gel electrophoresis. These properties were followed at the same time with an examination of the enzymatic properties of the enzyme.

The study of reassociation of subunits was followed using a stopped-flow fluorometer.

Major Findings: (See also Dr. Samuel Luborsky, NCI-5531). β-Galactosidase was found to dissociate in solutions of amines or of thiols, or at high pH.

L-Asparaginase is dissociated by relatively low concentrations of urea into its subunits. The exact concentration at which dissociation occurs is a function of the time during which the enzyme is exposed to the dissociating agent. In general the procedure has been to dialyze a solution of the enzyme against the appropriate concentration of urea for 16 hours at room temperature. Analytical ultracentrifugation showed that there was partial dissociation in 2.4 and 2.8 urea while at 3.2 M, dissociation was complete. Enzymatic activity paralleled these changes. Examination of the fluorescence properties proved to be a very useful method of studying the conformational changes accompanying dissociation. The native enzyme has its fluorescence maximum at 317 nm which indicates that the 4 tryptophans of this enzyme are located in a hydrophobic microenvironment. The fluorescence due to tyrosine has been quenched although it can be detected. The enzyme which has been partially dissociated shows a decrease in the 317 nm emission intensity accompanied by a slightly broadened half-band width. The completely dissociated enzyme at 3.2 M urea shows two fluorescent bands - one at 303 nm which is characteristic of tyrosyl residues and the other at 345 nm which is the wavelength at which tryptophanyl residues emit radiation when located in a hydrophilic environment. This indicates that the conformation of the protein has dramatically changed following dissociation.

At somewhat higher concentrations of urea, about 4.2 to 4.6 M, it is possible to follow that rate at which the tetramer dissociates by measuring the rate of decrease in the 317 nm fluorescence intensity. The fluorescence changes accompanying dissociation support the scheme that the subunits dissociate first and this is followed by the individual polypeptide chain unfolding to allow the tryptophan residues to be exposed to the outside environment. There is a very strong dependence on urea concentration so that dramatic changes occur over a narrow range of 0.2 M from 4.4 - 4.6 M.

Similarly the individual subunits in 4.5 M urea readily reassociate into the tetramer. The subunits in 4.5 M urea were mixed in the stopped flow fluorometer with an equal volume of 1.6 M sodium chloride. There is an immediate change from the spectrum having two emission peaks (303 and 345 nm) to the single emission band at 317 nm. This is then followed by the gradual increase in the intensity of the band until it reaches the intensity shown by the native molecule.

Dissociating agents such as SDS also cause a decrease in the intensity of the 317 nm band but this is not accompanied by exposure of the tryptophanyl residues to the hydrophilic environment. These results are in agreement with other observations on the mechanism of action of SDS.

Significance to Biomedical Research and the Program of the Institute: L-Asparaginase had found use in the treatment of acute lymphocytic leukemia and its effect presumably depends upon its ability to hydrolyze L-asparagine. One of the structural features that controls its catalytic function is the degree of association of its subunits into the tetramer. An understanding of the nature of the forces through which the subunits are aggregated might assist in modifications of the protein which will prevent rapid dissociation and prolong its catalytic activity.

Proposed Course: Further studies on the reassociation of subunits into the active tetramer are being studied.

Honors and Awards: None

Publications:

Shifrin, S., Luborsky, S. W., and Grochowski, B. J.: Inactivation and dissociation of β-galactosidase by amines. Biochem. Biophys. Res. Comm. 39: 997, 1970.

Shifrin, S., Grochowski, B. J., and Luborsky, S. W.: Dissociation of β-galactosidase by thiols. Nature 227: 608, 1970.

Serial No. NCI-5531
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Nature and aggregation of subunits of enzymes.

Previous Serial Number: Same

Principal Investigator: Samuel W. Luborsky

Other Investigators: Sidney Shifrin

Cooperating Units: None

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: β-Galactosidase and L-asparaginase were studied. Active L-asparaginase is known to be a tetramer. The subunits are inactive enzymatically. This study is designed to determine the properties of these enzymes by a variety of physical and chemical techniques and to study the relationship between structure and enzyme activity.

Methods Employed: Analytical ultracentrifugation: sedimentation velocity was used to monitor enzyme dissociation; polyacrylamide disc gel electrophoresis; dialysis; enzyme activity.

Major Findings: (See also Dr. Sidney Shifrin, NCI-5523.) β-Galactosidase was observed to dissociate in solutions of amines, thiols, or at high pH.

Native asparaginase from E. coli B exhibited concentration dependent sedimentation, with a $S^{\circ}_{20,w}$ of 8.6 s, in a sharp boundary consistent with a high degree of homogeneity. Polyacrylamide gel electrophoresis on the other hand revealed multiple bands in addition to the major component. The basis of this electrical heterogeneity is unclear. The enzyme has a broad pH -

sedimentation constant profile which parallels the activity versus pH profile. There is dissociation (to 1.2s) and a sharp drop in activity at pH 12 (irreversible), and dissociation (to 2.8s) and inactivation at pH 3 (almost completely reversible).

Activity and sedimentation are virtually unaffected by the presence of alkali metal ions in concentrations as high as 4 M, while 1 M divalent metal ions (Ca, Mg, Mn, Zn) cause reversible dissociation (to s < 2). Overnight dialysis against solutions of sodium lauryl sulfate (SLS) produced irreversible dissociation and inactivation above 0.1% SLS. However, below 0.1% SLS reversible inactivation occurs. Assays performed in 0.05% and 0.025% SLS yield no activity, but when the SLS is diluted away, full activity is restored. Polyacrylamide gel electrophoresis in SLS confirmed the dissociation of the enzyme to a subunit species of molecular weight 33,000.

Dialysis into urea solutions produced complete inactivation and dissociation between 2.8 - 3.2 M urea, which was reversible after diluting or dialyzing away the urea.

Significance to Biomedical Research and the Program of the Institute: Since the degree of aggregation of enzyme is often the basis of a cellular control mechanism of enzyme activity, understanding the type of subunits involved in the aggregation and their mode of interaction is of extreme importance. Moreover, since L-asparaginase possesses significant anti-lymphoma activity an understanding of its properties may help one to enhance this activity.

Proposed Course of the Project: The relationship between the active enzyme and its subunits will be explored further. The molecular weights of the enzyme and of its subunits produced in various ways will be determined.

Honors and Awards: None

Publications:

Shifrin, S., Luborsky, S. W., and Grochowski, B. J.: Inactivation and dissociation of β-galactosidase by amines. Biochem. Biophys. Res. Comm. 39: 997, 1970.

Shifrin, S., Grochowski, B. J., and Luborsky, S. W.: Dissociation of β-galactosidase by thiols. Nature 227: 608, 1970.

Serial No. NCI-5533
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project REport
July 1, 1970 through June 30, 1971

Project Title: Fine structure in the fluorescence spectrum of L-asparaginase.

Previous Serial Number: None

Principal Investigator: Sidney Shifrin

Other Investigators: Raymond R. Chen

Cooperating Units: Laboratory of Technical Development, National Heart and Lung Institute.

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: To examine the fine structure in the emission spectrum of L-asparaginase which is due to tyrosyl and tryptophanyl residues. High resolution analysis as well as low temperature studies permits a better understanding of the environment surrounding these aromatic residues.

Methods Employed: The spectra are examined with a modified Aminco Bowman spectrophotofluorometer fitted with a grating blazed at 300 nm and an 1P 28 photomultiplier which has a flat response from 250 nm to 400 nm. The fluorometer is equipped with a thermostated compartment which accommodates a short path length micro cuvette. The exciting beam is polarized to reduce scattering and the emitted beam is equipped with a 0.5 mm slit to resolve the fluorescent light. Solvent perturbation studies also allow conclusions concerning the nature of the environment around the tryptophanyl residues.

Major Findings: Native L-asparaginase in 0.05 M tris buffer, pH 7.4, shows unusual fine structure in its fluorescence spectrum. When the solution is excited at a wavelength of 275 nm both the

tyrosyl and tryptophanyl residues emit radiation at 303 nm and 317 nm, respectively. When the exciting light is at 295 nm, only the tryptophanyl residues absorb the radiation and the fluorescence spectrum has its maximum at 317 nm with a shoulder at 330 nm. These are unusual characteristics since solutions of tryptophan alone or tryptophan in proteins generally give broad featureless emission bands. This lack of spectral resolution presumably results from hydrogen bonding of the tryptophan imino group to the protonic solvent. The very low emission maximum (317 nm) as well as the fine structure suggests that the single tryptophanyl moiety in L-asparaginase is in an environment of low dielectric constant where hydrogen bonding to the medium cannot occur. Solvent perturbation studies are currently under investigation.

Significance to Biomedical Research and the Program of the Institute: The unusual physical properties of the tryptophanyl residue of L-asparaginase serves as a "reporter group" in following structural changes in the enzyme. Since these properties are characteristic of native conformations, predictions can be made as to whether or not certain chemical modifications alter the configuration of the individual subunit. Together with studies on the anti-lymphoma properties of the enzyme, it is anticipated that a correlation can be made between native structure and anti tumor properties.

Proposed Course of the Project: Further studies on the fluorescence properties of the native and modified enzyme are under way.

Honors and Awards: None

publications: None

Serial No. NCI-5534
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical modification of L-asparaginase.

Previous Serial Number: None

Principal Investigator: Sidney Shifrin

Other Investigators: Samuel W. Luborsky, Richard Adamson

Cooperating Units: Laboratory of Chemical Pharmacology, NCI

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objective: The purpose of this study is to chemically modify the structure of L-asparaginase by treatment with a variety of reagents and to test their effectiveness in the treatment of lymphomas.

Methods Employed: The enzyme has been treated with increasing quantities of succinic anhydride, dimethylaminonapthalenesulfonyl chloride, fluorescein isothiocyanate and tetranitromethane. The modified enzyme was examined by physico-chemical methods including ultracentrifugation, absorption and emission spectroscopy, polyacrylamide disc gel electrophoresis and by its enzymatic behavior.

Major Findings: (See Dr. Samuel Luborsky, NCI-5535.) Five mgs. of L-asparaginase treated with 3.76 mg. of succinic anhydride produces a slight change in the sedimentation velocity pattern of the enzyme and indicates that such treatment has produced partial dissociation accompanied by an equivalent loss in catalytic activity. Doubling the amount of succinic anhydride used in the treatment causes further dissociation together with further decrease in fluorescence activity, and increasing the amount of succinic anhydride to 18.8 mg. per 5 mg. of enzyme causes complete dissociation into subunits with no catalytic

activity. The fluorescence changes accompanying the dissociation also reveal that the protein conformation has changed and the single tryptophan residue present in the subunit is exposed to water.

These modified subunits are being tested in tissue culture for potential antilymphoma properties by Dr. Richard Adamson.

Succinic anhydride presumably reacts with the ε-amino group of lysine thereby changing the positively charged group of $\varepsilon\text{-}NH_3^+$ to the negatively charged succinyl derivative. Not all changes in the lysine group produce dissociation or enzyme inactivation. Treatment of the enzyme with dansyl chloride or with fluorescein isothiocyanate that react at the lysine residue produces a fluorescent derivative that is catalytically active and that retains its tetrameric structure. The fluorescence of tryptophan at 317 nm is partially quenched through energy transfer to the dansyl or fluorescein group. These derivatives are also being examined for antilymphoma properties by Dr. Adamson.

The nitrated derivatives are currently being prepared, purified and characterized.

Significance to Biomedical Research and the Program of the Institute: It is hoped that chemically modified L-asparaginase will possess antilymphoma activity and will remain at the site of action for prolonged times. If a monomer can be found to be active it has the advantage of not losing its function because of dissociation from the tetramer. It is also hoped that some chemically modified enzyme will not possess the toxic side effects now found with the native enzyme.

Proposed Course of the Project: To prepare further derivatives of the enzyme for physico-chemical characterization and pharmacological testing.

Honors and Awards: None

Publications: None

Serial No. NCI-5535
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Properties of L-asparaginase derivatives.

Previous Serial Number: None

Principal Investigator: Samuel W. Luborsky

Other Investigators: Sidney Shifrin, Richard Adamson

Cooperating Units: Laboratory of Chemical Pharmacology, NCI

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: To prepare and study the properties of various chemical derivatives of L-asparaginase; to study the relationship between structure, enzyme activity and antilymphoma activity.

Methods Employed: See Project NCI-5533. Also, tissue culture testing of the effects of these derivatives on tumor cells will be carried out.

Major Findings: Cf. also Dr. Sidney Shifrin, NCI-5533. Derivatives of L-asparaginase were prepared using increasing amounts of each of the following reagents: dimethylaminonaphalene sulfonylchloride (DNS), tetranitromethane (TNM), succinic anhydride (succ), and fluorescein isothiocyanate (Fl). At the levels of reagents used the Fl-asparaginase, though largely undissociated, was inactive, but all the other derivatives were either partially or fully active compared to unmodified control enzyme preparations. All DNS-enzyme preparations were undissociated and fully active. At higher levels of substitution, TNM-asparaginase lost some activity and was partially dissociated. This was also true for succ-enzyme, for which a good correlation was obtained between percent activity, percent tetramer, and percent succinylation.

Significance to Biomedical Research and the Program of the Institute: Since the degree of aggregation of enzyme is often the basis of a cellular control mechanism of enzyme activity, understanding the type of subunits involved in the aggregation and their mode of interaction is of extreme importance. Moreover, since L-asparaginase possesses significant antilymphoma activity understanding its properties may help modify these properties to enhance this activity.

Proposed Course of this Project: Other derivatives of asparaginase will be prepared. Molecular weight and other properties of the modified enzyme and its subunits will be determined. The antitumor activity of these derivatives will be determined.

Honors and Awards: None

Publications: None

Serial No. NCI-5503
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Properties of mouse leukemia virus RNA.

Previous Serial Number: Same

Principal Investigator: Samuel W. Luborsky

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/8
Professional:	1/8
Other:	0

Project Description:

Objective: To determine the size of the RNA in an RNA tumor virus. Recently an RNA-dependent DNA polymerase has been found in viruses in this group. There has been a previous suggestion of a subunit structure for these virus RNA molecules. The determination of an exact value for the molecular weight of tumor virus RNA molecules is a necessary part of our attempt to understand these properties.

Methods Employed: A 10X concentrate of mouse plasma containing the Rauscher leukemia virus was purified and concentrated using methods previously developed in this laboratory by differential centrifugation and isopycnic banding in a sucrose density gradient. The RNA was extracted by phenol after SLS and pronase treatments in the presence of bentonite and polyglucose sulfate. Equilibrium sedimentation studies were carried out in the analytical ultracentrifuge.

Major Findings: Sedimentation equilibrium distributions obtained in the analytical ultracentrifuge were recorded by scanning the ultraviolet absorption patterns at a wavelength of 265 nm. Most preparations were observed to be somewhat unstable

during the time necessary to achieve sedimentation equilibrium, and were partially degraded. It was possible to monitor such degradation by comparing successive patterns after equilibrium had been achieved and to find stable preparations. The average molecular weight obtained from many experiments was 11.2×10^6. This value is in agreement with those estimated by other less direct techniques, e.g., by calculation from sedimentation constant data using Spirin's empirical equation (as previously reported from this laboratory).

Significance to Biomedical Research and the Program of the Institute: This type of physical and biological characterization of murine leukemia viruses is fundamental to the characterization of the action mechanism of such viruses. More accurate information on the physical properties of nucleic acids of murine leukemia viruses and the clarification of the size, length, and single strandedness is necessary before meaningful studies can be undertaken on the mechanisms of replication and of transformation by such RNA viruses especially in the light of the suggestion that this RNA may have a novel structure and that these viruses possess RNA-dependent DNA polymerases.

Proposed Course of the Project: The accurate determination of the molecular weight completes the measurements currently contemplated on this system.

Honors and Awards: None

Publications:

Luborsky, S. W.: Sedimentation equilibrium analysis of the molecular weight of a tumor virus RNA. Virology, in press.

Serial No. NCI-5521
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Separation and characterization of rat liver mitochondrial DNA strands and of other biopolymers in density gradients.

Previous Serial Number: Same

Principal Investigator: Peter T. Mora

Other Investigators: Samuel W. Luborsky

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/8
Others:	1/8

Project Description:

Objectives: One objective of the project was to prepare mitochondrial NDA (M DNA) pure from nuclear DNA, to characterize the M DNA by physico-chemical techniques, to separate and analyze the two complementary strands, and to find out what is the likely base composition on one of the two strands which initiates RNA transcription on this so-called "sense" strand.

The other objective was to find a solute optimal for equilibrium density gradient separation in aqueous medium of sensitive biological macromolecules.

Methods Employed: In vivo and in vitro labelling of DNA by ^{3}H-thymidine. Cell homogenization and subcellular fractionation methods to obtain pure mitochondria. Electron microscopy, nucleic acid extraction and purification techniques, including hydroxyapatite chromatography. Characterization of the M DNA by physico-chemical, techniques, including specially devised analytical ultracentrifugation measurements. Preparative equilibrium density gradient ultracentrifugation techniques, using

radioactivity and biologic activities as markers. Spectroscopy methods on nucleic acids and on proteins.

Major Findings: a) Mitochondrial DNA Studies: Electron microscopically "pure" mitochondria were prepared from the liver of rats, pre-treated in vivo with ^{3}H-thymidine. The mitochondrial preparation was treated with DNase to degrade extraneous attached nuclear DNA. The mitochondria were then lysed with SLS and pronase, and the nucleic acids were extracted with phenol. Pure mitochondrial DNA was then obtained from the aqueous phase by preparative equilibrium density gradient centrifugation in neutral cesium chloride. This was followed by a preparative equilibrium density gradient centrifugation in alkaline cesium chloride, which resulted in two clearly separated DNA bands. The DNA from the two bands was collected, characterized in analytical ultracentrifugation experiments, and it was shown that they represent the complementary "light" and "heavy" strands. The interaction of the two strands with each other and also with synthetic polyrU and polyrG preparations was also studied. From such experiments it was concluded that the light strand is T and/or G poor and it has A and C rich clusters. Conversely, the heavy strand is T and/or G rich and has T and G rich clusters. In conjunction with very recent observations of others (Borst, Szybalsky) using similar mitochondrial DNA and also bacteriophage DNA, our work suggests that the T rich clusters on the heavy strand of the rat liver mitochondrial DNA are the initiation sites for RNA transcription, and this is probably the cause why the heavy strand is transcribed in vivo almost exclusively.

b) Studies on Media for Preparative Ultracentrifugation of Biological Macromolecules. We have surveyed the requirements for an ideal solute for density gradient ultracentrifugation of sensitive biopolymers, and concluded that the chemically synthesized highly branched polysaccharides, such as polyglucose, should best approach these requirements. We compared the density and the relative viscosity of solutions of polyglucose, sucrose, and of the natural sucrose polymer Ficoll. We also studied the behaviour of various polyelectrolytes in low viscosity polyglucose density gradients in equilibrium buoyant density measurement in the ultracentrifuge. Macromolecules or macromolecular complexes were shown to attain low apparent equilibrium buoyant density in polyglucose, probably because of an excluded volume effect of the solute. This allowed sedimentation to isopycnic position of complex biopolymers in the inert polyglucose solutions, which otherwise can be attempted only in concentrated solutions of heavy salts (such as CsCl or Cs_2SO_4). These salts, however, may salt out, or through osmotic effects may degrade or alter the properties of certain biologic macromolecular complexes. Density gradient centrifugation in polyglucose solution allowed separation of

electron-microscopically pure cell surface membrane components of various proteins and protein complexes.

Significance to Biomedical Research and the Program of the Institute: The role of mitochondrial DNA in higher organism is largely unknown, although indications are that it codes for structural proteins of cell membranes, and also that the amount of M DNA changes in embryonic differentiation. In this respect, cell surface changes might be related in part to the mitochondrial DNA also in malignant transformation. The control of the transcription of mitochondrial DNA thus may have some pertinence to regulation of cell growth through cell surface changes.

The most useful property of polyglucose as a density gradient solute was found to derive from the low osmolarity of its solutions, even at high concentrations. Polyglucose was found therefore especially useful when osmotic effects must be minimized: for example, when it was important to avoid damage to sensitive biological macromolecules or to macromolecular complexes, such as whole cells, viruses, or protein complexes. For example, there was an especially good recovery of murine leukemia virus infectivity after purification in polyglucose, as compared to purification in the commonly used solute gradients.

Proposed Course of the Project: The methodology developed both in the mitochondrial DNA strand fractionation and in the polyglucose density gradient experiments has been published. No further studies are planned explicitly in either fields, except when exploitation of such techniques is justified during experiments on other projects.

Honors and Awards: None

Publications:

Mora, Peter T., Leffler, Allan T. II, and Luborsky, Samuel W.: Properties of the complementary strands of a mitochondrial DNA. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 351: 955-960, 1970.

Mora, Peter T., and Luborsky, Samuel W.: Ultracentrifugation studies of polyelectrolytes in polyglucose density gradient. J. Macromolecular Science-Chemistry A4(5): 1067-1078, 1970.

Serial No. NCI-3101-D
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Nucleic acid homologies between normal rat liver and Morris hepatoma 5123.

Previous Serial Number: Same

Principal Investigator: Carl E. Smith

Other Investigators: Peter T. Mora

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To compare the RNA populations of liver and hepatoma with respect to nuclear and cytoplasmic components, and repeated and non repeated DNA sequences.

To examine liver and hepatoma chromatin for the possible presence of chromosomal RNA and chromosomal RNA-protein complexes having regulatory function.

To study the chromosomal proteins of liver and hepatoma.

Methods Employed: Fractionation of cell components; differential, analytical, sucrose gradient and equilibrium ultracentrifugation; spectrophotometry; radioactive labelling; methylated albumin-kieselguhr columns for nucleic acid fractionation, Nygaard-Hall (and modification of Gillespie-Spiegelman) introcellulose membrane technique for hybridizing nucleic acids, isolation and purification of DNA-dependent RNA polymerase, in vitro synthesis on DNA and chromatin templates of complementary RNA, sodium borohydride and dimethyl sulfate procedures for in vitro labelling of ribonucleic acid, base composition of RNA,

DEAE-Sephadex column chromatography.

Major Findings: A review of past work and preparation of manuscripts accounted for most of the year.

The method of preparation of chromatin from rat liver and from the minimal deviation hepatoma was presented, along with certain physico-chemical and biochemical properties of these chromatins. The hepatoma chromatin required greater care in purification and greater shearing for solubilization than liver chromatin. The chemical composition of the solubilized hepatoma chromatin, nevertheless, was in good agreement with that reported for chromatin from other rat liver tumors prepared in a somewhat different way. The hepatoma chromatin had higher RNA and total protein (but similar histone) content than liver chromatin.

Ultraviolet spectrum, thermal denaturation profile, the kinetics of in vitro incorporation into RNA of precursor nucleoside triphosphates with bacterial RNA polymerase, and the variation of incorporation with Mg^{++} and Mn^{++} ions were similar for the hepatoma chromatin as for the liver chromatin.

The base compositions of the RNA transcription products of DNA and chromatin templates obtained from hepatoma did not reveal large differences when compared to transcription products of DNA and chromatin templates obtained from liver. All RNA products had a high A+U/G+C DNA-like ratio, and a larger amount of GMP than CMP. The base composition of purified template DNA prepared from soluble chromatin as determined by spectrophotometric methods was the same for both hepatoma and liver, and was the same as reported previously for a representative rat tissue DNA employing chemical analysis.

Evaluation of the project's objectives in view of recent knowledge of mammalian RNA polymerases, chromatin structure, the expression of non-repeated DNA sequences and the fractionation of some histones into species showing differences with state of differentiation, as well as increasing evidences of post-transcriptional control lead us to conclude that while the project's objectives are still valid, both a more refined system(s) and a greatly restricted attention upon a single area mentioned above are in order.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the individual, basic components and structure of chromatin, and those molecules interacting with it, as well as the resulting RNA products should contribute to knowledge of cellular control mechanisms.

Proposed Course of the Project: The major findings on rat liver and hepatoma chromatin and on their transcription products have now been published. We plan to conclude the search for chromosomal RNA and RNA-protein complexes having regulatory function and submit our results for publication. Also other studies will be concluded on various factors given above.

Honors and Awards: None

Publications:

Smith, C. E., and Mora, Peter T.: Properties of chromatin from liver and from a chemically induced minimal deviation hepatoma of the rat. Biochim. Biophys. Acta 232: 643-650, 1971.

Serial No. NCI-5529
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: SV40 tumor-specific transplantation antigens (TSTA).

Previous Serial Number: Same

Principal Investigator: Richard W. Smith

Other Investigators: Peter T. Mora

Cooperating Units: Immunology Branch, NCI.

Man Years: (July 1, 1970 through June 30, 1971)

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: To develop in a model mouse system methods which would be applicable to future investigations of human TSTA: detection of TSTA by simple rapid assays, modification of such assays to permit assessment of the immunological interaction between tumor cells and the host, and use of such assays as a marker for the presence of the possible etiologic agents of cancer. Further objectives are solubilization and purification of TSTA, chemical analyses of the product, and assessment of its usefulness in immunoprophylaxis.

Methods Employed: Antiserum and lymphocytes were obtained from syngeneic mice immunized with SV40 transformed cells. Cell lines were maintained by standard tissue-culture methods. Other methods included cell cloning, in vivo and in vitro immunoselection, enzymic digestion of cell surface membranes, ion exchange and gel chromatography, and affinity chromatography. Assays for antigen were done in vivo and in vitro by serum cytolysis, cell mediated cytolysis, and lymphocyte blast transformation.

Major Findings: Serum mediated cytolytic assays for SV40 TSTA were developed. Solubilization and partial purification of the TSTA was accomplished by an enzyme digestion technique of the surface membranes of cultivated cells which allowed re-

tention of the viability of the cells. The usefulness of a partially purified soluble TSTA preparation in immunoprophylaxis was demonstrated.

SV40 TSTA was assayed by cytolysis-inhibition or absorption in several SV40 transformed mouse and hamster cell lines, in embryonic mouse tissue, and also in lytically infected green monkey kidney cells. TSTA was found in lytic infection, in SV40 transformed mouse cell lines, and in hamster cells containing SV40 T and S antigens and viral RNA. It was undetectable in hamster lines containing only S antigen but negative for T antigen and for viral RNA. It was likewise undetectable in early embryonic mouse tissue and a control hamster cell line.

SV40 transformed mouse cell lines are usually tumorigenic only in immunosuppressed syngeneic hosts, making in vivo assays difficult to interpret. By immunoselection procedures we have developed several SV40 transformed mouse cell lines which are highly malignant in immunologically competent adult animals, yet contain TSTA by in vivo and in vitro assays. One factor in the heightened malignancy is a decreased expression of cell surface TSTA on such cells.

Significance to Biomedical Research and the Program of the Institute: These studies provide methods for the study of virus-induced and other TSTA's, means for the assessment of host immune response to a tumor, and a means of measuring tumor cell surface antigen changes after growth in vitro and in vivo. They further demonstrate that in certain systems the specific immunity to tumors can be provided by immunization with soluble cell surface TSTA, providing ground-work for studies on human TSTA's.

Proposed Course of the Project: Continued biochemical work on the TSTA preparations in order to characterize TSTA and to separate it from transplantation antigens. Study of human cells transformed by SV40 and other viruses, and of human tumor cells.

Honors and Awards: None

Publications:

Smith, R. W., Morganroth, J., and Mora, P. T.: SV40 virus-induced tumor-specific transplantation antigen in cultured mouse cells. Nature 227: 141-145, 1970.

Serial No. NCI-5536
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: SV40 tumor-specific transplantation antigen expression in cells infected or transformed by mutants of SV40 virus.

Previous Serial Number: None

Principal Investigator: Richard W. Smith

Other Investigators: Dr. James Robb, Lab. of Molecular Biology, NIAMD

Cooperating Units: None

Man Years:

Total:	3/12
Professional:	2/12
Other:	1/12

Project Description:

Objectives: We are trying to determine the origin of SV40 TSTA and the requirements for its expression in lytic infection of green monkey cells, and in abortive and stable transformation of mouse cells, by using various temperature-sensitive mutants of SV40 virus.

Methods Employed: Standard virologic and tissue culture techniques are employed for cell growth, culture, and enumeration. Special microtechniques are employed to conduct experiments on a very small scale. Direct cytolytic and indirect cytolysis-inhibition assays for TSTA are performed using a newly developed method of great sensitivity. TSTA is also measured in vivo by immunization-challenge type protocols. The use of metabolic inhibitors and temperature sensitive mutants (permissive 32°, non-permissive 37°) permits evaluation of cell-virus interactions at the level of gene control.

Major Findings: SV40 TSTA is not detectable in lytic or abortive infection by direct serum-complement mediated cytolysis, but is detectable at very low levels by absorption of activity from dilute antisera. In two Swiss 3T3 mouse cell lines stably transformed by an "early function" SV40 mutant, TSTA does not modulate when cells are grown and assayed at the permissive temperature (T antigen 95% positive) or non-permissive temperature (T antigen <10% positive). This indicates that the genes controlling viral T antigen and the virus specific TSTA are not identical.

Significance to Biomedical Research and the Program of the Institute: These studies on virus-cell interactions and their modification by various viral mutations and various chemical inhibitors may elucidate the mechanism of the appearance of new surface antigenic changes on malignant cells.

Proposed Course of the Project: We plan to screen 24 temperature sensitive mutants of SV40 virus for temperature modulation of TSTA in transformation and in lytic infection, and attempt to determine the nature of viral gene or cellular gene function required for TSTA expression in malignant cells.

Honors and Awards: None

Publications: None

Serial No. NCI-5537
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cr^{51} release micro assays for measurement of histocompatibility antigens.

Previous Serial Number: None

Principal Investigator: Richard W. Smith

Other Investigators: None

Cooperating Units: Immunology Branch, NCI; Section on Lipid Chemistry, Lab. of Neurochemistry, NINDS

Man Years:

Total:	3/12
Professional:	2/12
Other:	1/12

Project Description:

Objective: The object of this project is to develop procedures for performing the Terasaki type of micro-cytotoxicity histocompatibility tests using radioactive Cr^{51} as the indicator of cell lysis. The ultimate goal is to develop the micro-technique into a highly versatile tool applicable to most clinical and research measurements of histocompatibility antigens.

Methods Employed: Standard tissue culture techniques are employed for cultivation of mouse, monkey, and human cell lines. In addition, special procedures for easy handling of many different cell lines were devised. Radioactive Cr^{51} is used to label tissue culture cells. Assessment of radioactivity is by standard liquid scintillation counting procedures. Antiserum is prepared by standard immunization procedures or is obtained from cooperating laboratories.

Major Findings: Murine epithelioid and fibroblastic cells, green monkey kidney cells, and human diploid fibroblast and continuous human lymphoid cell lines were labelled with radioactive Cr^{51}, while attached to the wells of 12 microliter Falcon

micro-test plates. The degree of radioactive labelling is several orders of magnitude higher than has previously been reported by any Cr^{51} labelling technique: up to 15,000 cpm per 1000 cells has been achieved. When three replicate microplate wells are counted together, the reproducibility is about plus or minus 10%. Histocompatibility antigens can be detected on human fibroblasts in microplate tissue culture using Cr^{51} release. When human lymphocytes in great excess were co-incubated simultaneously with specific HL-A typing serum and Cr^{51} labelled fibroblasts, Cr^{51} release was completely inhibited only if the inhibitor lymphocytes contain the HL-A antigen(s) common to the antiserum and target cells. Non-specific inhibition has not been detected up to a 400 to 1 ratio of inhibitor cells to target cells.

Significance to Biomedical Research and the Program of the Institute: The potential exists for a major improvement in histocompatibility typing procedures, permitting the assessment of very low levels of transplantation antigens which might be undetected by direct cytolysis.

Target cells are simple to handle, store and transport. Antisera used can be multispecific. A complete match can be performed on a single test plate.

In addition to providing a contribution to transplantation immunology research, these techniques are directly applicable to the study of certain tumor specific transplantation antigens in both experimental models and in the human.

Proposed Course of the Project: I propose to continue this work in order to standardize a method for measurement of histocompatibility and tumor specific antigens.

Honors and Awards: None

Publications: None

Serial No. NCI-5526
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Glycolipids in normal, DNA virus transformed and spontaneously transformed tumorigenic mouse cell lines.

Previous Serial Number: Same

Principal Investigator: Peter T. Mora

Other Investigators: Prof. Ingolf Dyong, Guest Worker, Mrs. Vivian W. McFarland, Dr. Richard W. Smith, LCB, NCI; Dr. Roscoe O. Brady, Jr., Dr. Federico A. Cumar and Dr. Peter H. Fishman, LNC, NINDS; Dr. K. K. Sanford, LBGY, NCI; Dr. James A. Robb, LMB, NIAMD.

Cooperating Units: Bionetics Research Laboratory, Bethesda, Md.

Man Years:

Total:	5 1/2
Professional:	3 1/2
Others:	2

Project Description:

Objectives: We have found recently a pattern of change in the amount of sialic acid containing glycolipids, the gangliosides, in cultivated mouse cells when such cells are transformed in culture by the tumor inducing DNA viruses polyoma and SV40. The change in ganglioside content was found to reflect mainly changes in cell membrane composition, and also it was found to occur hand in hand with the changes in the growth properties of cells in culture during the virus induced transformation. The objective was to confirm these changes in various cell lines after DNA virus induced transformations, to investigate whether such changes occur in spontaneous transformation of cells in culture, to obtain information on the chemical and the enzymological aspects of the changes, and finally to relate these observations to alteration of cell surface membranes of cells and to growth regulation of

cells in culture and in vivo.

Methods Employed: Tissue culturing methods (humidified CO_2 incubator, roller bottles, suspension cultures, etc.). Labelling of gangliosides, proteins, nucleic acids, etc., with radioactive precursors during cell growth in culture. Cell homogenization and subcellular fractionation techniques. Chemical isolation and characterization procedures, including thin-layer chromatography and gas-liquid chromatography. Radioactivity counting procedures.

Major Findings: a) Chemical Analysis (with Dr. Ingolf Dyong). The usual method of analysis of cell gangliosides consist of extraction of the glycolipids, separation of the various gangliosides by thin-layer chromatography and estimation of the gangliosides by colorimetry. The gangliosides can be tentatively identified by their migration rate on the thin-layer chromatogram, compared to authentic ganglioside samples. For definitive identification of gangliosides their carbohydrate constitution has to be determined. To do such determinations on the low amounts of gangliosides which are attainable from cultivated cells a very sensitive quantitation technique has to be developed. We were fortunate to have Prof. Ingolf Dyong to spend a year with us on sabbatical from the University of Münster, West Germany. Dr. Dyong, who is one of the leading carbohydrate chemists in Europe, developed here an appropriate chemical derivatization and gas chromatographic technique for the carbohydrate constituents of the gangliosides. Employing this direct chemical method, he compared three different established mouse cell lines with their SV40 virus transformed derivatives. The results were in line with our previous observations on changes in the gangliosides. In addition, the results provided quantitative information on the decrease in the G_{D1a} (disialotetrasaccharide ganglioside) content, and on the decrease in the precursor ganglioside homologs with shorter carbohydrate chains, supplying clues to the biochemical (enzymologic) mechanism.

b) Enzymology (with Drs. Brady and Cumar). Since the decrease in the ganglioside G_{D1a} was accompanied (in most of the cell lines) with a decrease of certain of its precursors with shorter carbohydrate chains, a crucial enzyme in the sequential synthesis of higher ganglioside: the enzyme which transfers N-acetylgalactosamine from uridine diphosphate N-acetylgalactosamine to hematoside (N-glycolylneuraminyl or N-acetylneuraminylgalactosylglucosylceramide) was suspect, and it was investigated in several normal and virally transformed derivative cell lines. Almost without exception, there was found a drastic decrease of this transferase activity, in both SV40 and polyoma transformed cells. This is the first biochemical change which was found to be common to both SV40 and polyoma viruses, and which pertains to cell membrane biochemistry, and not only to previously observed unspecific and

trivial changes which are naturally expected to accompany the higher rate of cell division after viral transformation (for example, increased DNA synthesis). Thus, this well defined enzymatic change provided an opportunity to begin clarifying biochemical changes, which while distal to the viral DNA, are common in both DNA virus-induced tumorigenic transformation, and are apparently pertinent to the regulation of the growth of the cells when in contact with each other.

c) Molecular Mechanism (with Dr. Cumar, Dr. Robb, and Mrs. McFarland). The repression of the enzyme uridine diphosphate N-acetylgalactosamine:hematoside N-acetylgalactosaminyl transferase, was found to be reversibly coupled to the changes in growth properties in tissue culture. That is, in the "flat" derivatives of the virus transformed cell lines which reverted to normal phenotypic growth property in culture, there was a trend of reversion in transferase activity near to that of the parent lines, in spite of the continued presence of viral DNA in these cells. Thus insertion of the virus genome into the cell genome is not sufficient: cells which are capable to repress the growth property alteration of the inserted virus genome (the phenotypically normally growing "flat revertants") also repress the biochemical change we found, the decrease in the transferase activity. Mouse cells productively infected with polyoma virus did not show the decrease in the transferase activity. Thus the enzyme change pertains only to DNA virus induced cell transformation and not to virus multiplication. Co-cultivation of enzymatically deficient virally transformed (non-permissive) cells with enzymatically active parental cells did not result in reduced enzyme activity in the enzymatically active cells, or in the restoration of enzyme activity in the virally transformed cells. Similarly, no change in enzyme activity occurred when admixing homogenates of the reciprocal cells. While the biochemical control of the transferase activity is not known, these biochemical findings are consistent with at least a partially common repressor mechanism in both SV40 and polyoma virus induced transformation of growth properties of mouse cells in culture. In temperature sensitive mutants of SV40 transformed Swiss or Balb/c mouse cells (with Dr. James Robb) there was no modulation observed in the transferase activity corresponding to that of the modulation of the T antigen.

d) Spontaneous Transformation of Cultured Cells (with Dr. K. Sanford and Dr. R. Brady). Mouse cells which were transformed "spontaneously" upon culturing in horse serum in Dr. Sanford's laboratory did not show any of the above biochemical differences, when compared to the non-malignant cells which were cultivated in fetal calf serum. Similarly, "spontaneous" transformation of certain other mouse cell lines (T AL/N) which occurred in our laboratory upon prolonged cultivation in fetal calf serum, did not

bring forth the biochemical changes in ganglioside metabolism, although the cells became highly tumorigenic in the syngeneic AL/N mouse. Currently other "spontaneously" transformed cell lines (for example, from Balb/c mouse) are being investigated. Up until now, when well controlled cell lines are compared, only the polyoma or SV40 transformed cell lines appear to exhibit the above described biochemical changes. Also no changes were observed in preliminary studies on cell lines transformed with RNA tumorigenic viruses (MSV and RSV) by Drs. Stuart Aaronson, John Bader and John Bassin (NCI, Etiology). Thus, at this stage of investigation it appears that the ganglioside changes occur only after transformation with the DNA tumorigenic viruses.

e) Other Biochemical and Morphological Studies on Cell Surfaces (with Drs. P. H. Fishman and R. W. Smith). The nature of the sialic acid incorporated into the cell surface membrane from radioactive sugar precursors is being studied with appropriate chemical and enzymological techniques. About half of the sialic (or neuraminic) acid appears to be in glycoproteins, the other half in glycolipids. Glycoprotein changes are not grossly apparent after SV40 virus induced transformation of cells, in contrast to the above discussed glycolipid changes. Digestion with neuraminidase liberates the sialic acid from glycoproteins, but apparently not in substantial amounts from the glycolipids in the cell membrane. Partial digestion of cell surface with proteolytic enzymes does not appear to alter the ganglioside biochemistry and pertinent enzymatic activity of normal or spontaneously transformed cells.

Studies of cell surface morphology by a modified phase microscopy (Nomarsky) technique and by electron microscopy (with the assistance of Mr. L. Waters), revealed an increased surface area and "roughness" of the surface of cells after the transformation with both SV40 and polyoma viruses and also after the spontaneous transformation in culture.

Significance to Biomedical Research and the Program of the Institute: Studies of cell surface biochemical changes which relate to loss of contact inhibition in culture and to acquisition of tumorigenic potential in vivo are of interest, especially when they may lead to understanding of changes in molecular mechanism (enzymology) which appears to be reducible to heritable changes on control processes on the DNA level in the virally induced transformation of cells.

Proposed Course of the Project: Special attention will be given to study the biochemical mechanism in the "flat" variant cells, since these cells are capable to repress the functioning of (viral genes) responsible for the altered cell growth property and at the same time repress the repression of the transferase

activity. There will be an attempt to correlate the transferase activity with other cell membrane biochemical changes (glycoproteins, virus induced tumor specific transplantation antigens as detectable by immune cytolysis technique (cf. NCI-5529)) and possibly relate these changes to changes in cell immunogenicity and in tumorigenicity in syngeneic mouse system.

Honors and Awards:

Participated in symposia by invitation: at the Vth International Symposium in Carbohydrate Chemistry, Paris, August, 1970; at the 8th International Biochemistry Congress, Switzerland, September, 1970; and at an International Symposium on Relationship Between Tumor Antigens and Histocompatibility System, Paris, February, 1971.

Publications:

Mora, P. T., Brady, R. O., and Smith, R. W.: Glycolipids in SV40 and polyoma virus transformed mouse cell lines. In Aminoff, D. (Ed.): Blood and Tissue Antigens. New York, N.Y., Academic Press, 1970, pp. 337-339.

Brady, R. O., and Mora, P. T.: Alteration in ganglioside pattern and synthesis in SV40 and polyoma virus-transformed mouse cell lines. Biochim. Biophys. Acta 218: 308-319, 1970.

Cumar, F. A., Brady, R. O., Kolodny, E. H. McFarland, V. W., and Mora, P. T.: Enzymatic block in the synthesis of gangliosides in DNA virus-transformed tumorigenic mouse cell lines. Proc. Natl. Acad. Sci. U.S.A. 67: 757-764, 1970.

Mora, P. T., Brady, R. O., and Smith, R. W.: Membrane biochemistry and antigenicity of spontaneously and virally transformed mouse cell lines. Immunochemistry 7: 867, 1970.

Mora, P. T., and Brady, R. O.: Changes in cell membrane biochemistry upon transformation in culture by tumorigenic DNA viruses. Transplantation Proceedings, in press.

Mora, P. T., Cumar, F. A., and Brady, R. O.: A common biochemical change in SV40 and polyoma virus transformed mouse cells coupled to control of cell growth in culture. Virology, in press.

Serial No. NCI-4833

1. Laboratory of Cell Biology
2. Ultrastructural Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electron microscope studies of normal, malignant and virus-infected cells and cell fractions

Previous Serial Number: NCI-4833

Principal Investigator: Dr. Nelson A. Wivel

Other Investigators:
Dr. Elaine A. Neale (staff fellow)
Dr. E. Kuff (NCI-366)
Dr. J. Rhim (Microbiological Associates Inc.)
Dr. G. Smith (NCI-3256)
Dr. A.M. Smith (East Carolina University)
Dr. W. Woods (NCI-4877)

Man Years:
Total - 2.25
Professional - 1.25
Other - 1.0

Project Description

Objectives:

The Ultrastructural Biology Section is concerned with the study of various factors which influence cell differentiation and malignant transformation. Particular emphasis is given to systems in which viruses represent the carcinogenic agent. The projects undertaken require the correlation of data from various disciplines such as electron microscopy, virology, immunology and biochemistry.

Methods Employed:

The principal methods involve the visualization of appropriate selected biological materials in the electron microscope, the use of tissue culture for propagation of viruses, the use of differential ultracentrifugation to isolate cell and virus fractions, and the detection of viral antigens by a variety of immunological techniques.

Major Findings:

1. A study of the pronephros of the carp, done in collaboration with Dr. M. Smith, has been completed. Ultrastructural studies of this fish organ suggest that it is possibly a primitive prototype of the mammalian lymph node.

There is good evidence of antibody production in the pronephros, and morphological evidence of plasmacytopoiesis, with several of the plasma cell precursors being similar to those forms characteristic of higher mammals.

2. A study with Dr. W. Woods has resulted in the characterization of a continous line of lymphoblastic cells growing in tissue culture. Two distinctive cell types were found in culture, the lymphoblasts which clearly were predominant, and giant macrophages (75-100 microns in diameter) which engulfed nonviable cells. These cells were derived from a spontaneous transplantable tumor of AKR mice, grew best in suspension culture, contained viral and cellular antigens of the Gross leukemia virus complex, produced infectious Gross virus, and exhibited tumorigenicity in AKR mice.

3. In a collaborative effort with Dr. J. Rhim it has been demonstrated that the long-term in vitro cultivation of Rauscher leukemia virus (RLV) in rat embryo cells is possible. Rat embryo cells initially infected with RLV have replicated infectious virus and produced complement-fixing antigen characteristic of the murine leukemia-sarcoma virus complex for more than 12 months. Production of complete virus was demonstrated by electron microscopy. When supernatant fluids from the eighth subculture of infected rat embryo cells were inoculated into newborn NIH Swiss mice, these animals developed a leukemia indistinguishable from the original disease described by Rauscher. The data indicate that rat embryo cells can be used for the cultivation of RLV in a carrier state.

4. A recent study of the distribution of intracisternal A-particles has revealed their presence in both normal and neoplastic tissues of five strains of mice and their hybrids. Cell types derived from all three embryonic germ layers contain these particles, and their presence in gonadal tissue of both sexes suggests that vertical transmission can take place. Such particles can coexist in the same cell with C particles and intracytoplasmic A-particles (mammary tumor virus precursors). Although the function of intracisternal A-particles has not been determined, they may represent the partial gene expression of one of the known oncogenic RNA viruses.

5. Further studies on the intracisternal A-particles in mouse plasma cell tumors are being continued with Dr. E. Kuff. It has now been established that the protein patterns, as seen on polyacrylamide gel electrophoresis, are similar in particles isolated from three separate transplant lines of BALB/c plasma cell tumors. These A-particles are deficient in the lower molecular weight proteins characteristic of murine leukemia viruses and their proteins fail to react on treatment with PAS under conditions where murine leukemia virus glycoproteins are clearly demonstrated. Several experiments strongly suggest that the group specific antigen of murine leukemia virus is not present in intracisternal A-particles.

1. Laboratory of Cell Biology
2. Ultrastructural Biology Section
3. Bethesda, Md.

6. A collaborative effort with Dr. G. Smith involves the isolation of intracytoplasmic A-particles (mammary tumor virus precursors) from spontaneous mammary tumors and transplantable Leydig cell tumors in C3H mice. The results to date indicate that these particles can be concentrated and isolated in a reasonably pure form. Through the use of such material it should be possible to answer important questions about the replicative cycle of mammary tumor virus. Intracytoplasmic A-particles from various types of tumors are being compared in terms of biological activity, biophysical properties and immunological profile.

7. Dr. Neale has completed a study on the effect of low temperature on the growth and fine structure of Bacillus subtilis. When logarithmically growing cultures were transferred from 37 to 15 C, the growth curves became atypical, and ultrastructural examination of the bacterial cells revealed deterioration of the mesosome and double cell wall formation. However, after a given time period, the cells regained the appearance of normal control cells, indicating a recovery from thermal stress. Subcultures of such cells continued to grow well at 15 C. Cultures transferred from 37 to 12 C did not show recovery from thermal stress, but cells grown at 15 C continued to grow at 12 C without further ultrastructural alteration. The results indicated that thermal stress induced structural alterations in this bacterial strain, that response to thermal stress could change during the period of stress, and that thermal history could affect the response to thermal stress.

Serial No. NCI-4833

1. Laboratory of Cell Biology
2. Ultrastructural Biology Section
3. Bethesda, Md.

Publications

Smith, M., Wivel, N., and Potter, M. "Plasmacytopoiesis in the Pronephros of the Carp, Cyprinus carpio." Anatomical Record 167: 351-370, 1970.

Woods, W., Wivel, N., Massicot, J., and Chirigos, M. "Characterization of a Rapidly Growing AKR Lymphoblastic Cell Line Maintaining Gross Antigens and Viral Replication." Cancer Res. 30: 2147-2155, 1970.

Rhim, J., Joglekar, M., Richardson, E., Wivel, N. and Huebner, R. "Long-Term in vitro Cultivation of Rauscher Leukemia Virus in Rat Embryo Cells." Proc. Soc. for Exp. Biol. Med. 135: 160-166, 1970.

Neale, E. K., and Chapman, G. B. "Effect of Low Temperature on the Growth and Fine Structure of Bacillus subtilis." J. of Bact. 104: 518-528, 1970.

Wivel, N. and Smith, G. "Distribution of Intracisternal A-Paritcles in a Variety of Normal and Neoplastic Mouse Tissues." Int. J. of Cancer 7: 167-175, 1971.

Serial No. NCI-5550
1. Laboratory of Cell Biology
2. Viral Oncogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on murine leukemia and sarcoma viruses

Previous Serial Number: Same

Principal Investigator: Kenneth S. S. Chang, M.D.

Other Investigator: Dr. L. W. Law (C:LCB-5553)

Man Years: Total - 2
Professional - 1
Other - 1

Project Description:

1. Studies on the genome activities of murine leukemia and sarcoma viruses in producer and nonproducer cells.

a) Effects of interferon. ---This is to investigate the possibility of the presence of an interferon-sensitive stage of murine sarcoma virus (MSV) genome expression in the nonproducer cells of hamster tumor (HT-1) induced by MSV; and to study the possible difference between the state of integration of viral genome in nonproducer hamster tumor cells and that in the murine leukemia virus (MuLV) carrier cell lines as reflected by interferon sensitivity.

Preliminary results indicate that the activity of MuLV in the carrier cell line is suppressed by interferon as revealed by decreased ability of the treated cells to produce virus and to effect an MSV genome rescue activity. By contrast, pretreatment of HT-1 cells with hamster interferon could not suppress MSV retrieval when the treated cells were co-cultivated with MuLV-carrier cells. This may be indicative of the absence of viral messenger RNA formation in HT-1 cells before trigger mechanism for MSV genome rescue is turned on, or that the viral messenger RNA contains a cellular messenger RNA which allows a normal attachment and readout at the ribosomal sites of the interferon-treated cells for translation.

b) Effects of UV-and X-irradiation on the helper activity of Rauscher leukemia virus (RLV).

The nonproducer hamster tumor cell line (HT-1) which contains MSV genome was superinfected with a highly purified and concentrated RLV preparation. By using this technique which allows a direct MSV genome rescue, and by UV- or X-irradiating the RLV before its use as the superinfecting virus, it is possible to analyze the size of RLV genome which is responsible

for helper activity. The infectivity of the irradiated RLV decreased logarithmically as a function of the dose of radiation, while the RNA-dependent DNA polymerase activity remained unchanged or slightly increased. Preliminary results with helper activity indicated that UV-irradiation inactivated the latter at the same rate as that of infectivity. X-inactivation of the helper activity is being investigated.

2. Studies on MSV (0).

MSV (0) is a murine sarcoma virus carried by a rat tumor (MSB-1). Although the tumor was originally induced by MSV (MLV), MSV (0) produced by this tumor no longer infects mouse cells. Unsuccessful attempts were made to adapt this virus back to mouse cells by infecting the latter with the cell extract of MSB-1. In order to effect an efficient infection, the MSB-1 cells were UV-irradiated to kill the cells without affecting much the titer of MSV (0) and mixed with mouse embryo cells. After cloning, the mouse cells which contain no free rat cells were shown to produce MSV (0). However, the possible presence of rat-mouse hybrid cells cannot be excluded. Use of rat- and mouse-interferons to differentially affect production of MSV (0) in these cells is being attempted. Chromosome analysis of the cloned cells will be done.

Such an adaptation procedure is also being conducted in vivo. The mixture of BALB/c mouse embryo cells and UV-irradiated MSB-1 cells was injected into newborn BALB/c mouse and the resulting tumor was passaged from mouse to mouse.

It is hoped that by such an alteration of host specificity, the nature of defectiveness of MSV (0) may be elucidated.

A highly pruified and concentrated preparation of MSV (0) is being secured, and with this material, an immunologic and physicochemical characterization of MSV (0) will be conducted.

3. Isolation of producer and nonproducer cell clones of an hemangioma induced by MSV (MLV) in (C57BL x C3H) F1 mice.

Clones were isolated from the hemangioma which produced either murine leukemia virus as revealed by XC test, or murine sarcoma virus as revealed by focus formation on mouse embryo cells, or none. Attempts are being made to characterize the leukemia and sarcoma viruses, and to rescue MSV genome which may be present in the nonproducer clone.

Immunological studies made on all of these clones indicate the presence of an antigen which manifested its activity principally by immunological enhancement in (C57BL x C3H)F1 mice immunized with MSV (MLV) or by temporary growth and surgical removal of the neoplastic cells themselves.

Serial No. NCI-5550

1. Laboratory of Cell Biology
2. Viral Oncogenesis Section
3. Bethesda, Md.

4. Characterization of viruses isolated from SJL, and Figge mouse strains, and those from plasmacytomas of BALB/c mice.

Viruses were isolated from the organ extracts of these mice by *in vitro* cultivation, and detected by XC test. Immunological and biological activities of these viruses are being investigated.

Serial No. NCI-5550
1. Laboratory of Cell Biology
2. Viral Oncogenesis Section
3. Bethesda, Md.

Publications:

Chang, S.S., Gilden, R.V., and Huebner, R.J.: Rescue of murine sarcoma virus genome in mixed cultures of 'non-producer' hamster tumour cells and helper virus carrier cell lines.
J. Gen. Virol. 10: 107-110, 1971.

Law, L.W. and Chang, S.S.: Effects of antilymphocyte serum (ALS) on the induction of lymphocytic leukemia in mice.
Proc. Soc. Exp. Biol. Med. 136: 420-425, 1971.

SUMMARY STATEMENT
LABORATORY OF MOLECULAR BIOLOGY
GL&C, NCI
July 1, 1970 - June 30, 1971

This is the first annual report of the Laboratory of Molecular Biology. We have initiated studies in three general areas.

1. How is the expression of genetic information controlled in both bacterial and animal cells?

2. What is the molecular basis of genetic recombination? The work on the molecular basis of genetic recombination is directed by Dr. M. E. Gottesman who was recently appointed Chief of the Biochemical Genetics Section.

3. Are some of the properties of cancer cells due to alterations in their metabolism of cyclic AMP?

Regulation of gene expression in E. coli by cyclic AMP - I. PASTAN AND COLLABORATORS. Over the past 70 years it has been established that E. coli make various inducible catabolic enzymes at a greatly diminished rate when grown on glucose. About three years ago Perlman and Pastan showed that this deficiency in enzyme synthesis was due to low levels of cyclic AMP in the cell and that glucose acted by lowering cyclic AMP levels. When cyclic AMP was added to cells repressed by glucose, enzyme synthesis was restored and in the case of the lac operon lac mRNA synthesis was stimulated.

A class of mutants was isolated which were unable to make β-galactosidase or other inducible enzymes despite high cyclic AMP levels. These were shown to be missing a protein which binds cyclic AMP, the cyclic AMP Receptor Protein (CRP). This protein has been purified to homogeneity by W. Anderson who is currently engaged in characterizing it.

Using this protein B. de Crombrugghe and B. Chen have shown that CRP, RNA polymerase and lac DNA are the essential macromolecular elements which together with cyclic AMP are needed to achieve controlled production of lac mRNA. Cyclic AMP and CRP are required for RNA polymerase to form a preinitiation complete with lac DNA. Lac mRNA synthesis is repressed by lac repressor and repression is overcome by appropriate inducers. This is the first time controlled transcription of a bacterial gene has been achieved in a completely defined system.

Another operon under cyclic AMP control is that concerned with galactose metabolism. J. Parks developed a cell-free system of E. coli which makes the enzyme galactokinase when supplemented with λpgal DNA and cyclic AMP. With appropriate mutants he was also able to show that CRP was required for galactokinase synthesis.

With λpgal DNA as a template P. Nissley then showed that together with cyclic AMP the macromolecular elements required for gal mRNA synthesis are CRP and RNA polymerase. Like the lac system, cyclic AMP and CRP function to allow RNA polymerase to form a preinitiation complex with DNA. When nucleoside triphosphates are added, gal mRNA is made.

Regulation of expression of biotin operon - K. KRELL AND M. GOTTESMAN.

In contrast to the operons under the control of cyclic AMP, biosynthetic operons are not. K. Krell and M. Gottesman have chosen to study the biosynthetic biotin operon which lies adjacent to the integration site of phage λ and for which λbio transducing phages are available. They have begun first to study the control by lambda genes of the expression of biotin genes and have found that there is a stop signal in the b_2 region of the λ chromosome which appears to prevent RNA polymerase which has initiated transcription within the lambda genome from transcribing into the biotin genome.

Integration and Excision of Phage λ - M. SHULMAN AND M. GOTTESMAN.

They have begun to characterize the lambda attachment sites and isolate point mutants in the attachment site. The isolation of these mutants has allowed them to conclude that mutations that affect the capacity of a phage for excision also affects its capacity for integration.

Cyclic AMP and Cancer - I. PASTAN AND COLLABORATORS.

Within the last year we have begun to investigate the possible role of altered cyclic AMP metabolism in cancer. This work was initiated by G. Johnson who found that when transformed cells were treated with dibutyryl cyclic AMP or cyclic AMP and theophylline, they grew more slowly, regained the spindly shape characteristic of untransformed cells and adhered more tightly to the substratum. For cyclic AMP to cause this effect protein synthesis was required. Prostaglandin E_1 a substance which activates adenyl cyclase has an effect similar to that of dibutyryl cyclic AMP.

More recently studies on cyclic AMP metabolism in cells transformed by various oncogenic viruses have been initiated. When mouse cells are transformed, there are changes in both adenyl cyclase activity and cyclic AMP levels. Thus various aspects of cyclic AMP metabolism are altered in some types of cancer.

Serial No. NCI-8000
1. Lab. of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Gene Expression in E. coli by cyclic AMP.

Previous Serial Number: None

Principal Investigator: Ira H. Pastan

Other Investigators: Benoit de Crombrugghe, Beatrice Chen, John Parks, S. Peter Nissley, Wayne Anderson, Maxwell Gottesman, Robert L. Perlman and Michael Emmer

Cooperating Units: Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases

Man Years (July 1, 1970 through June 30, 1971)

Total:	6 9/10
Professional:	6 4/10
Other:	5/10

Project Description:

Objective: Cyclic AMP is required for the synthesis of a variety of enzymes in E. coli, most of which are inducible. Cyclic AMP acts at a transcriptional level. Our objective is to determine how cyclic AMP controls gene activity and how cyclic AMP levels are controlled.

Methods Employed: Studies on mRNA and enzyme synthesis have been carried out in intact cells and in cell-free extracts. Mutants which are defective in cyclic AMP metabolism have been isolated and characterized.

Major Findings: We have previously been shown that the repression of the synthesis of inducible enzymes by glucose is due to a fall in cyclic AMP levels and that cyclic AMP overcomes the repression by glucose of the synthesis of β-galactosidase, galactokinase and other inducible enzymes. We have isolated mutants unable to synthesize cyclic AMP due to a deficiency in adenyl cyclase (cya^-). Such mutants are unable to synthesize inducible enzymes unless supplemented with cyclic AMP. We have also isolated mutants (crp^-) deficient in a cyclic AMP receptor protein (CRP). These mutants are also unable to synthesize inducible enzymes even when cyclic AMP is present.

To determine in intact cells if cyclic AMP acted at transcription we

measured the effect of cyclic AMP on the rate of synthesis of lac and gal specific mRNA. We found that cells with low cyclic AMP levels made very little of each of these mRNAs, and the addition of cyclic AMP greatly increased mRNA synthesis. However, mutants deficient in CRP were unable to make lac or gal mRNA even if cyclic AMP was present at high levels. We were also able to show that cyclic AMP promoted lac mRNA synthesis in cells in which protein synthesis was arrested by chloramphenicol. To do this we used cells which carry a mutation (SuA) which makes them deficient in the breakdown of mRNA, since in chloramphenicol-treated cells, mRNA not associated with ribosomes is rapidly broken down.

To study the details of cyclic AMP action we set up a cell-free system (S-30 described by G. Zubay and coworkers) which catalyzes the synthesis of β-galactosidase when supplemented with DNA containing lac genes (supplied by a lac transducing phage, λh80dlac) and cyclic AMP. We then showed that extracts from the crp^- mutant were unable to catalyze β-galactosidase synthesis unless supplemented with highly purified CRP (see below).

We have developed a lac mutant which makes unusually large amounts of β-galactosidase, apparently due to a mutation in the lac promoter which allows the lac genes to be transcribed more efficiently. DNA from this mutant (λh80dlac p^S) is a more efficient template than DNA from a normal lac operon.

Employing λh80dlac p^S DNA we have measured lac transcription in unfractionated extracts of E. coli. By conventional fractionation methods we prepared a soluble fraction which is eluted from ribosomes with 0.5 M KCl and catalyzes cyclic AMP-dependent lac transcription. In extracts from a crp^- mutant, CRP is also required. Lac mRNA synthesis is repressed by lac repressor and repression overcome by the inducer IPTG. From those experiments it appeared that the minimum requirements for in vitro control of lac mRNA synthesis include lac DNA, RNA polymerase, CRP and cyclic AMP, lac repressor and an appropriate inducer.

We then showed that controlled lac transcription could be carried out in a system containing two highly purified proteins, RNA polymerase and CRP, together with λh80dlac DNA, cyclic AMP, and nucleotides. In these experiments also, transcription was repressed by lac repressor and repression overcome by appropriate inducers. Apparently cyclic AMP, CRP, RNA polymerase and lac DNA form a preinitiation complex at the lac promoter and lac transcription begins when nucleotides are added.

As a supplementary approach to an understanding of cyclic AMP's action and the regulation of gene transcription studies were also begun in cell-free extracts on gal operon expression.

Galactokinase synthesis was demonstrated in a cell-free system which carries out coupled transcription and translation of bacteriophage λpgal DNA. The enzyme produced in vitro resembled galactokinase produced in vivo in its substrate specificity, divalent cation requirements, molecular weight and

resistance to inactivation by toluene. Deoxyribonuclease, ribonuclease and chloramphenicol prevented enzyme synthesis. Galactokinase synthesis was stimulated up to 12-fold by the addition of cyclic AMP. This stimulation required cyclic AMP receptor protein and could be prevented by the addition of cyclic GMP. Synthesis of galactokinase, unlike synthesis of β-galactosidase, was inhibited by guanosine 3'-diphosphate, 5'-diphosphate.

Also, a transcription system was developed using λpgal DNA as template and E. coli RNA polymerase. The gal specific mRNA was measured by prehybridizing ^{3}H-RNA product with λ DNA and then performing a second hybridization with separated strands of λpgal DNA. Addition of cyclic AMP and CRP to this system caused a 15-fold increase in gal specific mRNA hybridizing to the correct strand of λpgal DNA (ℓ-strand), whereas there was no increase in the small basal level of ^{3}H-RNA hybridizing to the incorrect strand. Experiments with rifampicin showed that cyclic AMP and CRP promote the formation of a rifampicin-resistant preinitiation complex.

The achievement of controlled lac and gal transcription in a defined system has been due to our ability to prepare homogeneous CRP free of nucleases or other proteins which bind to DNA and interfere with transcription. When examined by ultracentrifugal methods, CRP appears to be composed of two identical subunits, and the molecular weight of the mononer is 22,500. The amino acid analysis of CRP is consistent with this structure. By equilibrium dialysis the binding of cAMP to CRP was found to be reversible ($K_d = 1.4 \times 10^{-5}$ M). Cyclic GMP which inhibits cyclic AMP's action on transcription, competitively inhibits the binding of cyclic AMP ($K_i = 0.7 \times 10^{-5}$ M).

Significance: In cancer cells genes which are normally quiet become active due to some alteration in the control of gene expression. It is not yet possible to study the expression of individual genes in animal cells. Therefore our studies on the control of the transcription of lac and gal genes by cyclic AMP, cyclic AMP receptor protein, and lac repressor are a model on which to build an understanding of how gene expression is controlled in higher organisms.

Proposed Course: 1. We have now developed a system which permits us to begin to examine the chemical mechanism by which cyclic AMP and CRP divert RNA polymerase to initiate gene transcription.

2. To determine the sequence of the initial portion of lac and gal mRNA and thereby establish if the promoter or operator regions of the gene are transcribed.

3. To search for other regulatory molecules which control gene expression.

4. To determine to architecture of the genetic regions where gene transcription is initiated.

Honors and Awards: None

Publications:

Varmus, H., Perlman, R., and Pastan, I.: Regulation of lac Messenger RNA synthesis by cyclic 3'-5' Adenosine Monophosphate and Glucose. J. Biol. Chem. 245: 2259-2267, 1970.

Friedman, R.M., and Pastan, I.: Mechanism of action of 2-Mercapto-1 (ß-4-pyridethyl) benzimidazole: A reversible inhibitor of interferon activity. Proc. Nat. Acad. Sci. 65: 104-109, 1970.

De Crombrugghe, B., Varmus, H.E., Perlman, R.L., and Pastan, I.: Stimulation of lac mRNA Synthesis by Cyclic AMP and Cell Free Extracts of Escherichia Coli. Biochem. Biophys. Res. Commun. 38: 894-901, 1970.

Pastan, I. and Perlman, R.L.: The role of adenyl cyclase and cyclic Adenosine 3',5'-Monophosphate in biological systems. An international colloquium sponsored by the Fogarty International Center to be published by the Government Printing Office, in press, 1971.

Emmer, M., de Crombrugghe, B., Pastan, I. and Perlman, R.L.: The Cyclic AMP Receptor Protein of E. Coli: Its Role in the Synthesis of Inducible Enzymes. Proc. Nat. Acad. Sci. 66: 480-487, 1970.

Pastan, I. and Perlman, R.L.: Cyclic Adenosine Monophosphate in Bacteria. Science 169: 339-344, 1970.

Pastan, I., Pricer, W., and Blanchette-Mackie, J.: Studies on an ACTH Activated Adenyl Cyclase from a Mouse Adrenal Tumor. Metabolism 19: 809-817, 1970.

Varmus, H.E., Perlman, R.L., and Pastan, I.: Regulation of lac transcription in Escherichia coli by cyclic 3',5'-adenosine monophosphate: Studies with DNA-RNA hybridization competition. J. Biol. Chem. 245: 6366-6372, 1970.

Perlman, R., Chen, B., de Crombrugghe, B., Emmer, M., Gottesman, M., Varmus, H.E., and Pastan, I.: The regulation of lac transcription by cyclic adenosine 3',5'-monophosphate. Cold Spring Harbor Symp. Quant. Biol. 35: 419-423, 1970.

Pastan, I. and Perlman, R.L.: Cyclic AMP in Metabolism. Nature - New Biology 229: 5, 1971.

De Crombrugghe, B., Chen, B., Gottesman, M., Pastan, I., Varmus, H.E., Emmer, M., and Perlman, R.L.: Regulation of lac mRNA synthesis by cyclic AMP, cyclic AMP receptor protein and lac repressor in a soluble cell-free system. Nature - New Biology 230: 37-40, 1971.

Varmus, H.E., Perlman, R.L., and Pastan, I.: Lac transcription in antibiotic-treated E. coli: Regulation by cyclic AMP and pseudo-polar effects of chloramphenicol and puromycin. Nature - New Biology 230: 41-44, 1971.

Pastan, I., Perlman, R.L., Emmer, M., Varmus, H.E. de Crombrugghe, B., Chen, B.P., and Parks, J.: Regulation of gene expression in Escherichia coli by cyclic AMP. Recent Prog. in Hormone Res., in press, 1971.

Miller, Z., Varmus, H.E., Parks, J.S., Perlman, R.L., and Pastan, I.: Regulation of gal messenger ribonucleic acid synthesis in E. coli by 3',5'-cyclic adenosine monophosphate. J. Biol. Chem., in press, 1971.

Parks, J.S., Gottesman, M., Perlman, R.L., and Pastan, I.: Regulation of galactokinase synthesis by cyclic adenosine 3',5'-monophosphate in cell-free extracts of E. coli. J. Biol. Chem. 246: 2419-2424, 1971.

Pastan, I., de Crombrugghe, B., Chen, B., Anderson, W., Parks, J., Nissley, S.P., Straub, M., Gottesman, M., and Perlman, R.L.: Regulation of gene expression in E. coli by cyclic AMP. Proc. Miami Winter Symposia 1971. (Eds.): Ribbons, D.W., Woessner, J.F., and Schultz, J., North-Holland Publishing Co., Amsterdam, The Netherlands, in press, 1971.

Serial No. NCI-8001
1. Lab. of Molecular Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Role of Cyclic AMP in the Regulation of Growth and Morphology of Transformed and Normal Fibroblasts

Previous Serial Number: None

Principal Investigator: Ira H. Pastan

Other Investigators: George Johnson, Charles Peery, Jacques Otten and Massimo d'Armiento

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:	5 1/10
Professional:	4 1/10
Other:	1

Project Description:

Objective: To investigate how cyclic AMP regulates the growth and morphology of normal and transformed fibroblasts, and to determine the nature of the alteration of cyclic AMP metabolism in transformed fibroblasts.

Methods Employed: Cell culture and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules.

Major Findings: When various lines of transformed fibroblasts are treated with dibutyryl cyclic AMP or cyclic AMP and theophylline, they acquire some of the morphologic and growth characteristcis of normal cells. Cell growth is slowed, the cells adhere more tightly to the substratum, become more spindly in appearance and tend to line up in parallel arrays. Similar responses occur with human osteosarcomas and a human melanoma. The morphologic response is inhibited by puromycin but not actinomycin D, suggesting new protein synthesis but not new RNA synthesis is required. Colchicine also blocks the response; thus microtubular proteins have a role in the process. Dibutyryl cyclic AMP accentuates the normal spindly appearance of untransformed fibroblasts, but has no effect on the morphology of a variety of normal and transformed epithelial cells, suggesting that other mechanisms probably operate in epithelial cells.

Significance: Cyclic AMP appears to be able to cause a phenotypic

reversion of transformed cells and cyclic AMP metabolism may be altered in certain types of cancer.

Proposed Course: We plan to investigate if some of the altered growth properties of cancer cells are due to low cyclic AMP levels, and to determine the nature of the defect in cyclic AMP metabolism in cancer.

Honors and Awards: None

Publications:

Johnson, G.S., Friedman, R.M., and Pastan, I.: Restoration of normal morphology in sarcoma cells by cyclic AMP and N^6-butyryl cyclic 3',5'-adenosine monophospate. Proc. Nat. Acad. Sci. 68: 425, 1971.

Johnson, G.S., Friedman, R.M., and Pastan, I.: Cyclic AMP treated sarcoma cells acquire several morphological characteristics of normal fibroblasts. Proc. N. Y. Acad. Sci., in press, 1971.

Serial No. NCI-8002
1. Lab. of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Analysis of the fusion of XC cells induced by homogenates of murine leukemia virus-infected cells and by purified murine leukemia virus.

Previous Serial Number: None

Principal Investigator: George Johnson

Other Investigators: Ira H. Pastan and Robert M. Friedman

Cooperating Units: Laboratory of Pathology, National Cancer Institute

Man Years (July 1, 1970 through June 30, 1971)

Total:	3/10
Professional:	3/10
Other:	0

Project Description:

Objective: To characterize the mechanism by which cells producing murine leukemia virus promote the fusion of XC cells.

Methods Employed: Various cells and MuLV have been grown in tissue culture.

Major Findings: XC Cells, a RSV-induced rat sarcoma, undergo syncytium formation in the presence of murine leukemia virus (MuLV) infected cells. (Klement et al, PNAS 3, 1969). We have shown that intact, infected cells are not required, but that infected cell homogenates and also purified MuLV are effective in promoting the cellular fusion. For fusion to occur a lipoprotein appears to be required; however, the specific synthesis of macromolecules by the XC cells is not necessary.

Significance: Cell fusion does not require the contact of living cells but can be promoted by factors contained within the free MuLV virus.

Proposed Course: Discontinued.

Honors and Awards: None

Publications:

Johnson, G.S., Friedman, R.M., and Pastan, I.: Analysis of the fusion of XC cells induced by homogenates of murine leukemia virus-infected cells and by purified murine leukemia virus. J. of Virology, in press, 1971.

Serial No. NCI-8003
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Integration and Excision of Phage λ.

Previous Serial Number: None

Principal Investigators: Marc Shulman and Maxwell E. Gottesman

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:	1.5
Professional:	1.5
Other:	0

Project Description:

Objectives: To study the mechanisms involved in integration and excision by phage λ.

Methods Employed: Standard microbiological techniques and published modifications developed in this laboratory have been employed.

Major Findings: We have begun to characterize the λ attachment sites, the regions of DNA which are the substrate of integration and excision of phage λ. To study these regions we are isolating phage which have point mutations in the attachment sites. To isolate these mutants we employ an unusual transducing phage which carries the two hybrid attachment sites and is thus itself a substrate for intramolecular recombination promoted by the site-specific phage excision system.

Our preliminary findings so far can be summarized: (1) Mutations which affect the capacity of the phage for excision also affect its capacity for integration. (2) The attachment sites might contain internally a short region which is equivalent in the different attachment sites.

Significance to Bio-medical Research and the Program of the Institute: These studies elucidate the mechanisms of phage integration and excision. These mechanisms may be of importance in a variety of virus-host stystems.

Proposed Course: We will isolate more mutants which have altered attachment sites and then try to correlate the map position, genetics, and various phenotypic expressions of the mutations.

Honors and Awards: None

Publications:

Shulman, Marc and Gottesman, Maxwell E.: λatt^2: A transducing phage capable of intramolecular Int-Xis promoted recombination. In Bacteriophage Lambda, A. D. Hershey (Ed). In press, 1971.

Serial No. NCI-8004
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Control by lambda genes of the expression of bacterial genes.

Previous Serial Number: None

Principal Investigators: Kenneth Krell and Maxwell E. Gottesman

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:	1.5
Professional:	1.5
Other:	0

Project Description:

Objectives: To characterize the mechanisms by which lambda genes control the expression of bacterial genes, specifically those of the biotin operon.

Methods Employed: Standard microbiological and biochemical techniques as well as those developed by the principal investigator and in this laboratory.

Major Findings: When normal lambda prophage is induced, the genes of the adjacent galactose operon are expressed at levels comparable to those found in the presence of an inducer such as D-fucose. Phage mediated galactose operon expression is dependent upon phage replication. The biotin operon is located on the right of the prophage. There has been no report to date of biotin operon expression as a result of prophage induction. The only data available suggest that DNA replication does not occur in the bio region upon induction of a normal lambda prophage.

a. Desthiobiotin synthetase activity has been detected, under conditions of repression, when a lambda prophage deleted for the b_2 region is induced. This appears to be a site specific effect. No biotin enzyme is detected when the b_2 region is present. Galactose enzyme expression is observed in both cases.

b. The rate of enzyme synthesis is linear over a 40 minute period.

c. Biotin operon expression is only partially dependent on phage replication.

d. The appearance of enzyme is totally dependent on phage late function control, i.e., lambda gene Q. Superinfection of a Q^- lysogen with a heteroimmune Q^+ phage elicits desthiobiotin synthetase expression.

These results suggest that a termination function exists in the b_2 region of the lambda genome. Whether this effect is upon transcription or translation has not yet been determined. If the effect is upon transcription then read-through of the biotin operon can occur even in the presence of the biotin-repressor complex if initiation has occurred at an upstream promoter site. Perhaps the biotin-repressor complex partially masks the normal initiation site thus preventing polymerase binding.

Significance to Bio-medical Research and the Program of the Institute: These studies involving a system in which a virus supervenes in the control of a host function may help to elucidate virus-host interactions in other systems.

Proposed Course: The nature of the b_2 termination function will be further characterized by studies of biotin operon transcription and the site of the termination function mapped by genetic techniques.

Honors and Awards: None

Publications: None

SUMMARY STATEMENT
Laboratory of Pathology
General Laboratories and Clinics
National Cancer Institute
July 1, 1970 to June 30, 1971

Introduction

Approximately a year ago the Laboratory of Pathology was reorganized into several sections. The sections of human diagnostic pathology which were formerly designated the Pathologic Anatomy Branch are included in this reorganization which is listed by sections in the report below. The professional staff of each of these sections is also listed followed by a brief summary of their diagnostic and research activities.

Many members of the staff, in addition to their laboratory work, serve on national and international committees, editorial boards, etc. Some of the principal activities of this sort are: Dr. Stanton, Scientific Editor of the Journal of the National Cancer Institute; Dr. Dawe, Associate Editor for International Journal of Cancer and Cancer Research; Dr. Rabson, Associate Editor of JNCI and Cancer Research; Dr. Grimley, Associate Editor of JNCI; Dr. Friedman, Associate Editor of Journal of Virology; Dr. O'Conor, Chairman of the UICC Commission on Epidemiology; Dr. Thomas and Dr. O'Conor, WHO collaborating centers for classification of neoplasms; Dr. Berard, participation in Tutorial on Neoplastic Hematopathology; and Dr. Dawe, Chairman of Committee on Comparative Oncology, UICC. Other committees, symposia, etc., participated in by the staff are listed in the annual report.

The staff of the Laboratory presented a scientific program to the Board of Scientific Counselors at its meeting in Bethesda on March 22-23, 1971.

Dr. Dawe and Dr. Berard have made extensive plans for a week-long workshop on Comparative Hematopoietic and Lymphoreticular Neoplasms in animals and man which will be held in Bethesda May 10-14, 1971.

Dr. Katherine Herrold retired in January 1971. Dr. Roger O'Gara died of an acute myocardial infarction on January 25, 1971.

The staff of the Laboratory of Pathology were either senior authors or co-authors of 82 papers published in 1970.

Surgical Pathology Section:

Dr. O'Conor, the residents and other pathologists participating in the diagnostic services of the Laboratory of Pathology are responsible for the diagnostic reports in Surgical Pathology. Approximately 5,553 specimens were accessioned. These include approximately 2,500 bone marrows. Dr. O'Conor and the residents regularly make rounds with Dr. Ketcham and the surgeons in the Surgery Branch of the NCI and show most of the surgical biopsies and operative specimens at the weekly Surgical Pathology Conference.

A number of studies on human disease were published during the year and other studies now going on will lead to publications. These, in part, include

evaluation of serum alpha fetoprotein in USA and African patients with primary liver cancer (O'Conor), study of immunologic stimulation as a factor in Burkitt's tumor (O'Conor), study of patients with advanced lymphosarcoma treated by radiation therapy and study of a hepatocellular carcinoma with the carcinoid syndrome (O'Conor). Dr. Powell is continuing the clinicopathological study of pulmonary involvement by Hodgkin's disease and has a publication on the experimental induction of interstitial emphysema. Papers by the residents included studies on biogenic amines in a variety of human tumors including medullary carcinoma of the thyroid gland and a non-chromaffin paraganglioma (DeLellis), studies on the spleen in Type I hyperlipoproteinemia (Buja) and a variety of cardiovascular lesions including stenosis of the pulmonary artery in a patient with dissecting aneurysm (Buja), clinicopathologic correlation of liver biopsies in patients with Hodgkin's disease (Roth), synovial sarcoma of the larynx (Roth), fetal toxoplasmosis in a patient infected by a leukocyte transfusion (Roth), and excess lipofucsin in constitutional hepatic dysfunction (Barth). Some of the residents also published on a variety of projects in laboratory experimental pathology.

Dr. O'Conor's electron microscopy laboratory was remodeled and put into operation and now allows him to work on a number of projects including EM studies of human and animal tumors including in vivo and in vitro studies, in collaboration with Dr. Rabson, of the tumors produced by Herpes saimiri.

Postmortem Section:

Dr. Thomas, the residents and other staff pathologists were responsible for 279 autopsy examinations. A gross review is held with the residents and clinical staff each morning at 8:30 a.m. and the staff and residents present selected autopsy findings at numerous clinical-pathologic conferences. At present, approximately 30 scientists in the various laboratories have requested particular tissues from the surgical pathology and autopsy services. Human material has been used for a number of studies including a retrospective study of more than 400 cases of Hodgkin's disease (Thomas and Berard), incidence and significance of thrombi in coronary arteries of patients with myocardial infarction (Roberts and Buja), histochemical and EM studies of the myocardium (Buja, Ferrans) and central nervous system complications in chronic myelogenous leukemia (DeLellis). Dr. Buja has also collaborated in a number of studies on the heart of experimental animals.

Miss Bowling and her staff continued to provide excellent histological slides and special stains for the human tissues. Mr. Edwards, assisted by Mr. Banks, took approximately 2,500 color photographs of the gross lesions in human tissues.

Cytopathology Section:

Dr. Malmgren, Dr. Chu, Dr. Berard and the residents were responsible for the cytopathology interpretation of human material. Approximately 4900 specimens were accessioned and reported. Dr. Chu has established a service laboratory for cytogenetics and efforts are underway to evaluate methods for automating cytogenetics. Studies by this staff included immunologic factors in malignant melanoma (Malmgren), alteration in sex chromosomes in females as it may

relate to cancer (Chu) and an evaluation of aspiration biopsies of breast lesions (Malmgren and Chu). Dr. Malmgren's work in immunology included studies of human neoplasms and their reaction to their own serum with immunofluorescence and to the demonstration of a common antigen in a number of sarcomas and melanomas (in collaboration with Dr. Morton).

During the year Dr. Malmgren initiated a contract with the AEC group at Los Alamos, New Mexico to study the automation of cytology.

Ultrastructural Pathology Section:

Dr. Grimley and his staff are responsible for examination of human tissues, teaching of residents and independent research. Three main areas of experimental work are: (1) the study of anti-viral effects of rifampicin; (2) study of the role of cell structures in arbovirus development; and (3) study of endoreticular tubules observed in lymphoma cells and in the glomerular epithelium of patients with systemic lupus erythematosus. The latter research has been performed by Dr. Pincus (in collaboration with Dr. Grimley). Dr. Pincus joined the staff on July 1, 1970 for a period of one year. Dr. Donald Henson will be joining Dr. Grimley's staff on July 1, 1971.

Hematopathology Section:

Dr. Berard, Dr. Krueger and the residents and other staff participants are responsible for a variety of special studies of patients with leukemia, malignant lymphoma and immunoproliferative diseases. These include studies on Hodgkin's disease (Berard and Thomas), Burkitt's tumor, childhood and adult lymphosarcoma (Berard), graft-versus-host syndrome (Krueger and Berard) and macroglobulinemia (Berard). Experimental studies (Krueger and Berard) include investigation of the production of malignant lymphomas in mice with immunosuppression and antigenic stimulation, studies of dogs with bone marrow grafts, and assessment of the toxic effects of chemotherapeutic drugs.

Dr. Berard conducts a weekly hematopathology-clinical conference and with Dr. Thomas participates in a staging conference of the malignant lymphomas.

Viral Oncology and Molecular Pathology Section:

Dr. Friedman has one Guest Worker (Dr. Judith Levin) and one Visiting Scientist (Dr. Yun) working with him during this past year. Dr. Friedman and his staff have continued their studies on interferon and have localized the action of interferon to the step between transcription and translation of virus genetic information. Methods for the super-induction of interferon have been studied and interferon production in a cell-free system are also being studies. Dr. Levin and Dr. Friedman have completed an analysis of arbovirus RNA forms. Dr. Yun is working on a project studying the effect of ribonuclease on the morphology and biochemical reactions of tumor cells.

During the coming year, Dr. Friedman will be on overseas assignment in England and in Israel.

Dr. Phillip Permiman, a Senior Staff Fellow, started working with Dr. Rabson in January 1971.

The major objective of Dr. Rabson's experimental work has been to identify and characterize human oncogenic viruses. He and his associates have previously worked with a number of model systems but are now concentrating on isolation attempts with human tumors and intensively studying one animal model system-malignant lymphoma induction in New World primates by Herpes saimiri. They have isolated a lymphoid cell line from a lymphoma of the marmoset induced by H. saimiri and have been studying the virus-cell relationship in this system. The virus seems to be present in all the lymphoid cells in some latent or repressed form and the cells are capable of continued rapid multiplication. When these cells are placed in contact with monkey kidney cells in culture they cease to multiply and they release H. saimiri which then destroys the monkey kidney cell monolayer. The mechanisms of this "induction by contact" are under study and other methods of induction are also being examined. These induction methods will then be applied to human malignant lymphoma cells.

Comparative Oncology Section:

Dr. Dawe, Dr. Banfield, Dr. Snell, Dr. Stanton, Dr. Dunham and Dr. Mulay are the professional staff members of thie Section. Dr. Takayama is a Guest Worker with Dr. Dawe.

For a number of years the experimental pathology conducted by the members of this Section was restricted almost entirely to laboratory rodents. During the past few years the scope of investigative work has broadened to include animals at many phylogenetic levels from poikilothermic vertebrates to primates.

In preparing the report for the Section, Dr. Dawe describes the experimental work in three categories: (1) tumor characterization; (2) mechanisms involved in tumor development; and (3) development and application of methods for the detection of carcinogens. In the first category he cites the description of carcinoid tumors in Mastomys which is associated with a syndrome similar to the Zoolinger-Ellison syndrome by Dr.Snell; the description of spontaneously occurring tumors in Mastomys of the prostate gland, rete testis and seminal vesicles (Snell); study of fibrous long spacing collagen and spiney-collagen associated with a variety of neoplasms and in cardiac conditions (Banfield); EM study of neoplasms in oysters and mussels (Banfield); demonstration of a herpes-type virus in a fatal infectious disease in oysters (Banfield); description of epizootic lymphosarcoma in northern pike (Dawe); use of marker chromosomes to prove the epithelial origin of salivary gland tumors induced by polyoma virus (Dawe); and studies of time-lapse phase-contrast motion pictures of the effects of dibutyryl cyclic AMP on L-cells derive from mice (Morgan and Dawe in collaboration with Drs. Johnson and Pastan). Under category 2 is listed Dr. Stanton's work on the mechanisms of asbestos carcinogenesis and the development of a laboratory model for pulmonary carcinogenesis with tobacco products; Dr. Dunham's experiments to test the influence of environmental factors (plant extracts from Curacao, alkaloids from the betel quid, plastic tubing to produce partial obstruction

and stasis of food and ingested material in the esophagus) on mouth and esophagus cancers in animals; and Dawe's experiments to test the concept that cancer cells are autonomous because they do not require certain growth stimulating substances which are apparently supplied to normal cells by adjacent stroma and mesenchyme. In the third category are cited the studies on diet and chemical carcinogens (Mulay); use of the electron probe and other specialized instruments to locate metals and other elements in cells and tissues (Banfield); experiments using organ cultures to develop methods for assaying chemical carcinogenicity (Takayama); and use of filter-feeding and bottom-feeding fish and other aquatic animals as indicators of environmental carcinogen (Dawe and Committee on Comparative Oncology of the UICC).

Several members of the Section are preparing chapters for the Tumor Atlas being edited by Dr. Turusov (International Agency for Research on Cancer) and it is expected that these atlases will become standard references for experimental oncologists. Dr. Stanton, a member of Dr. Turusov's Editorial Board, is preparing a chapter on bone tumors in mice; Dr. Snell on adrenal gland neoplasms in rats; and Dr. Dawe on salivary gland neoplasms in mice.

Pathological Technology Section:

Miss Betty Sanders and her staff continue to provide superior service to the members of our staff and a large number of investigators in the National Cancer Institute.

Serial No. NCI 500
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Histogenesis and Pathology of Induced and Spontaneous Tumors of Laboratory Animals

Previous Serial Number: Same

Principal Investigator: Katharine C. Snell, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Others:

Project Description:

A. Praomys (Mastomys) natalensis: (Mastomys)

Objectives: (1) To study and describe the tumors and other lesions that develop spontaneously, some of which appear to be counterparts of human diseases. (2) To ascertain the mechanism of the effect on host animals bearing transplantable carcinoids that originated within the glandular stomach. (3) To study the enterochromaffin and enterochromaffin-like cells of the gastrointestinal tract of normal Mastomys and of those that have primary gastric carcinoids.

Methods Employed: (1) Most Mastomys are allowed to live as long as possible in order to furnish information on tumor incidence. Prior to necropsy, blood serum from each animal is collected and frozen. This will be used to study the immunoglobulins in animals with pyelonephritis, the relationship of autoantibodies to striated muscle in animals with thymomas, and the histamine content in animals with carcinoids of the glandular stomach. With Dr. Carel Hollander of Rijswijk, The Netherlands, a description of tumors of the male sex organs is being written. This paper will combine the findings in his colony and the one at NIH.

(2) Transplanted carcinoid tumor tissue and blood serum from Mastomys bearing these transplants is harvested, frozen, and shipped to Dr. Syun Hosoda of Nagoya, Japan for estimation of histamine and histidine decarboxylase activity.

(3) By the use of the Faxitron following instillation of barium by stomach tube, we are selecting Mastomys with probable gastric carcinoids. A number of these animals, along with controls, will be shipped to Dr. Rolf Håkanson of Lund, Sweden and also to Dr. Enrico Solcia of Pavia, Italy. These investigators will study the origin of the carcinoids (probably from enterochromaffin-like cells) and the enterochromaffin and enterochromaffin-like cells of the normal gastric mucosa.

Major Findings: (1) Malignant argyrophilic carcinoids occur in approximately 1/2 of Mastomys 18 months of age or older. Thymomas and thymic hyperplasia appear in a high percentage of animals, particularly in females, and are often associated with myositis and myocarditis. Carcinosarcomas of the seminal vesicle occur in a small number of animals. Carcinoma of the rete testis, an unusual tumor in human beings, is found in an appreciable number of old males.

(2) Mastomys bearing transplanted argyrophilic carcinoids develop multiple ulcers of the stomach and duodenum. Pathologic changes similar to those of human malabsorption syndrome are seen in the jejunum and ileum. The transplanted tumors do not contain or produce measurable amounts of gastrin. They do contain appreciable amounts of histamine, specific histidine decarboxylase and L-amino acid decarboxylase, which may be related to the inherent properties of the parent cells, probably histamine-containing argyrophil cells, distributed in the gastric mucosa.

(3) No results as yet.

Significance: Mastomys are becoming of increasing importance as experimental animals, since in many ways their diseases parallel those of man (gastric carcinoids, osteoarthritis, glomerulonephritis, multiple endocrine adenomas, for example). These animals may be useful for determination of the chronologic and functional relationships between thymoma and anti-striated muscle antibodies, and possibly polymyositis and myasthenia gravis. It seems likely that study of their gastric carcinoids may shed light upon the relationship between various types of enterochromaffin cells, pluriglandular adenomas and the production of histamine. The continued study of untreated Mastomys is important in order to establish a baseline for future experimental work.

Proposed Course: Continue the projects currently planned or in progress. During the next year or two we hope to complete necropsies and histologic study on at least 500 untreated Mastomys and to prepare a monograph on their tumors and other lesions. A workshop on Mastomys is tentatively planned for the coming year.

B. Untreated Rats

Objectives: To determine the incidence of tumors and other lesions that occur spontaneously in rats of 6 inbred strains and in a group of germ-free rats.

Methods Employed: In the early part of this experiment rats were serially killed at 3-month intervals. That part has been completed and we are now adding data on rats allowed to live as long as possible as well as on old germ-free rats.

Major Findings: None other than reported for 1970.

Significance: The knowledge gained from a study of neoplasms and other lesions that occur spontaneously in laboratory animals can provide a basis and possible analogy to the study of the tumors and other lesions that occur in human beings.

Proposed Course: Continuation of the study of untreated and germ-free rats as old animals become available. From our material a chapter of the IARC Atlas on Tumors of Laboratory Animals is being prepared in collaboration with Dr. Carel Hollander. This chapter will illustrate the various types of tumors of the adrenal cortex and medulla.

C. Mechanism of Fetal Resorption Following Treatment of Pregnant Rats with the Polypeptide, Nisin, or Some of its Fractions

Objectives: To study the changes that take place in the fetus and in the uterus and other organs of pregnant rats treated with nisin or its fractions. This is a collaborative study with Dr. Erhard Gross of NICHD.

Methods Employed: Dr. Gross treats pregnant rats with various doses of the compound under study, by various routes, and on various days of pregnancy. We perform the necropsies with him and examine the histologic sections.

Major Findings: Nisin or its fractions appears, under certain circumstances, to cause partial or complete fetal resorption. It is too early to conjecture about the mechanism. The fractions, however, especially when given intravenously, causes severe damage to the kidneys as evidenced by precipitation of the compound within the renal tubules.

Significance: The ultimate aim of the group in NICHD is probably the discovery of various drugs that can be used in human population control. If so, it is highly desirable that they be aware of the potential hazards as well as the mechanism of action.

Proposed Course: Continuation of necropsies and examination of histologic sections.

D. Monkeys

Objectives: Originally the objective of this experiment was to determine whether 3-methylcholanthrene injected into the wall of the stomach would induce gastric carcinoma. Since, after many years of observation, no gastric cancers have been found, we are keeping the remaining monkeys to study any tumors or other lesions that may occur with age.

Methods Employed: 3-methylcholanthrene was injected or implanted in the wall of the stomach of rhesus monkeys 18 to 19 years ago. The monkeys, which are now 23 or more years old are being observed and allowed to live as long as possible. Prior to necropsy, skeletal surveys will be made by Dr. Doppman of the Clinical Center x-ray department. Also, brains will be perfused by Dr. Henryk Wisniewski of the Albert Einstein College of Medicine, who is interested in studying the aging process of neurons by electron microscopy.

Major Findings: Nothing significant has been observed in the stomach of any monkey necropsied. Three cases of spinal ankylosis, bilateral carcinoma of the kidney, diabetes with hyalinization of the islets of Langerhans, and adenocarcinoma of the ascending colon are the principal pathologic findings so far observed.

Significance: With the increasing use of monkeys in cancer research it is important to study old animals to determine the incidence of spontaneous disease. Very few colonies of monkeys of known and advanced age are in existence so that our animals constitute an important group for future study. Hyperostotic spondylosis is a disorder that is currently the subject of considerable clinical study. These monkeys may offer an opportunity for observing changes in bone which are almost never available in human autopsy material. The finding of carcinomas of the kidney and of the colon as well as the hyalinized islets in the diabetic monkey suggest that monkeys, if allowed to live long enough, may indeed develop many of the same diseases found in man.

Proposed Course: Continued observation of monkeys with necropsies as necessary with collaborative studies as indicated under Methods.

E. Transplantation of Tumors

Objectives: To carry interesting or unusual tumors in transplant for possible future study in this laboratory or by other investigators.

Methods Employed and Major Findings: The following tumors are carried by subcutaneous, intraperitoneal, or intramuscular injection:

494..Adrenal cortical carcinoma, OM/N rat. Tumor has been used by several investigators for hormone research and is currently being studied by Dr. Max Sweat of the University of Utah, and by Dr. Evan Cameron of the Welsh National School of Medicine, Cardiff, Wales, and in tissue culture by Dr. L.M. Edelstein of the New England Medical Center, Boston.

555..Gastric adenocarcinoma induced by 2,7-FAA in ACI/N rat. Tumor develops metaplastic bone, bone marrow and cartilage in center.

328..Gastric adenocarcinoma, C57BL mouse, induced by intramural injection of 3-MCA into the wall of the stomach. Mice in which this tumor has grown for a long time develop hypervolemia. Currently being studied is tissue culture by Dr. L.M. Edelstein of New England Medical Center, Boston.

586..Skin appendage tumor induced in ACI/N rat by oral administration of 2,7-FAA, being studied by Dr. Shu Wen How and in tissue culture by Dr. L.M. Edelstein of the New England Medical Center, Boston.

645,673,680,691..Malignant argyrophilic carcinoids that originated in the glandular stomach of untreated Mastomys.

648..Hepatic cell carcinoma that originated in a Buffalo rat that received aflatoxin in alcohol intragastrically by stomach tube.

Significance: The study of transplantable tumors continues to be a useful tool for investigators interested in hormone research, tissue culture, biochemistry, and transformation of tumor type during successive transplant generations.

Proposed Course: To maintain the tumors in transplant and make them available to other investigators.

Publications:

1. Hosoda, S., Nakamura, W., Snell, K.C., and Stewart, H.L.: Histamine production by transplantable argyrophilic gastric carcinoid of Praomys (Mastomys) natalensis. Science 170: 454-455, 1970.

2. Stewart, H.L., Dunn, T.B., and Snell, K.C.: Proliferative lesions of the lungs of mice. In Nettesheim, P., Hanna, M.G., and Deatherage, J.W. (Eds.): Morphology of Experimental Respiratory Carcinogenesis. Oak Ridge, Tenn., USAEC Division of Technical Information Extension, 1970, pp. 161-184.

3. Hosoda, S., Nakamura, W., Snell, K.C., and Stewart, H.L.: Histidine decarboxylase in the transplantable argyrophilic gastric carcinoid of Praomys (Mastomys) natalensis. Biochem. Pharmacol. 20: In Press, 1971.

Serial No. NCI 501
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Carcinogenesis in the Cheek Pouch of Hamsters or in the Esophagus of Hamsters and Rats: A Search for Factors in the Environment that may be Related to Cancers in Man

Previous Serial Number: Same

Principal Investigator: Lucia J. Dunham, M.D.

Other Investigators: Roger W. O'Gara, M.D. (Deceased)

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives: To identify substances or factors in the human environment that induce or influence the development of cancers.

Methods Employed: 1) Several plants used for herb teas (Annona, Heliotropum, Krameria, Acacia) under consideration as possible causes of cancers of the esophagus in Curacao, NWI, are being assayed in the hamster cheek pouch or esophagus with and without added injury to tissues by calcium hydroxide (lime).

2) Arecoline, an alkaloid present in the betel quid used in Southeast Asia, is being assayed in hamsters by oral administration 2 times per day, 5 days per week.

3) The abdominal portion of the esophagus of hamsters or rats is chronically constricted by means of surgically placed cuffs of polyethylene tubing, and carcinogenesis is attempted by means of intra-esophageal instillation of benzpyrene.

4) L.J. Dunham serves as consultant in experimental pathology to contract proposal submitted to Carcinogenesis Section by Dr. G. Kapadia, Department of Pharmacology, Howard University. The purpose is to conduct biologic screening of selected plants from Curacao. This work was initially planned in part by the late Dr. Roger W. O'Gara. Dunham serves also as a consultant in connection with the current posthumous studies of plant materials conducted in the laboratory of Dr. O'Gara.

Major Findings: 1) In an earlier experiment, Annona produced advanced atypia in a cheek pouch and an esophagus of hamsters, and Heliotropum apparently produced an esophageal papilloma. An experiment is under way involving intensive treatment of these sites with Annona and Heliotropum, and also with Krameria and Acacia which were not previously tested at these sites, but which produce sarcomas at subcutaneous sites in rats. The experiments have been under way about half of the two year period of observation.

2) The experiments with arecoline will be continued for at least another year. An esophageal papilloma and a thyroid carcinoma developed in a pilot experiment with arecoline.

3) An experimental group of hamsters with constricting esophageal cuffs and subjected to 1 or 2 instillations of benzpyrene in dimethylsulfoxide is under observation. An experiment is being started with a similar group subjected to a larger number of instillations of benzpyrene, in 70% alcohol instead of dimethylsulfoxide. These will probably be long-term experiments.

Significance: The frequencies of cancer in the human mouth and esophagus vary widely by geographic area, and the ratios by sex vary also. Constrictions, dilatation, esophagitis, and scarring are among precursor lesions of cancer of the esophagus that have been reported. Environmental factors may be important in the genesis of this cancer, and also in cancer of the mouth which is suspected of being related to such chewing habits as betel quid, or nass, or tobacco. There is believed to be an urgent need for methods of assaying the carcinogenic effects of environmental factors.

Proposed Course: An experiment is undertaken to confirm the observation of advanced hyperplastic lesions produced by selected plant materials, and to intensify or hasten the development of these lesions. It is proposed to extend the experiment to other suspected carcinogens in the human environment, and to continue attempts to develop a reliable method of testing the effects of suspected weak carcinogens on mucous membranes of hamsters.

Constriction of the esophagus in hamsters and rats simulates in animals a situation suspected of influencing the development of some esophageal cancers in man. If baseline studies yield sufficient useful data, it is proposed to extend the experiments to testing suspected carcinogens in plants and chemical fractions thereof.

Serial No. NCI 501a
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Geographic Pathology Field Study on Uterine Cancer in Negro Women in New York City and the Washington, D.C. Metropolitan Area

Previous Serial Number: Same

Principal Investigators: Lucia J. Dunham, M.D., Louis B. Thomas, M.D., and Harold L. Stewart, M.D.

Other Investigators: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/2
Other: 0

It is anticipated that the article will be submitted for publication this calendar year.

Serial No. NCI 501b
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Review of the Scientific Literature on the Subject of "Cancer in Man at the Site of Prior Benign Lesion of Skin or Mucous Membrane"

Previous Serial Number: Same

Principal Investigator: Lucia J. Dunham, M.D.

Other Investigators: None

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives and Methods Employed: To categorize the prior benign lesions: (1) Induced by known carcinogens (2) Benign lesions of a familial disease (3) Congenital lesions (4) Benign tumors (5) Lesions based on trauma or inflammation (6) Idiopathic lesions found clinically associated with cancer (7) Lesions that are manifestation of a focal neuropathy or constitutional disorder.

Major Findings: The types of injury and the pathology of the lesions are noted and discussed. Risks of cancer are described by the type and site of lesion.

Significance: To improve understanding of the natural history of some human cancers and to provide suggestions for research studies from such data.

Proposed Course: The manuscript is being typed in its final form, and will be submitted for publication.

Serial No. NCI 501c
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Gambier, an Ingredient of the Betel Quid, Administered to Mice by Various Routes

Previous Serial Number: Same

Principal Investigators: Calum S. Muir, M.D. and Lucia J. Dunham, M.D.

Other Investigators: None

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Other:

Project Description:

Gambier was administered to about 500 C_3H/HeN and hairless mice by subcutaneous route, by painting and inunction, and in food and drinking water.

Tumors were not produced during the course of experiments carried out at the National Cancer Institute. Muir and Hwang, working in Singapore, have recently produced sarcomas at subcutaneous sites in mice by repeated injections of an alkaline alcoholic extract of Gambier. Dunham has tried to produce sarcomas in the loose areolar tissue beneath the pouch epithelium and also in the tissues beneath the skin of the dorsum of the hamster foot by repeated injections of an alkaline alcoholic extract of Gambier. The experiment is completed and tumors were not produced.

Serial No. NCI 501g
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Report on Histologically Diagnosed Cancers in Indians of the United States, 1950 to 1966

Previous Serial Number: Same

Principal Investigators: Gert L. Laqueur, M.D., John C. Bailar, M.D., and Lucia J. Dunham, M.D.

Other Investigators: None

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Other:

Project Description:

The histologic and site diagnoses of 694 cancers in USA Indians will be reported. Dunham and Bailar will prepare an article for publication from a current draft of the report. This should soon be in final form.

Serial No. NCI 504
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An Electron Optical Investigation of Normal and Pathologic Tissue

Previous Serial Number: Same

Principal Investigator: William G. Banfield, M.D.

Other Investigators: George Kasnic, Clyde Dawe, Willie Morgan, Austin Farley, and Alan Rabson

Cooperating Units: Biological Laboratory, National Marine Fisheries Service (NOAA), Oxford, Maryland

Man Years (July 1, 1970 - June 30, 1971)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: 1. To describe and interpret the ultrastructure of selected pathologic states and to compare the results with suitable controls.

2. To look for and study the inciting agent or accompanying agent in neoplastic and other disease states using human, animal and tissue culture material.

Major Findings: 1. A study of the dense granule occurring in the elementary body of the pox viruses.

The small, dense granule first described by us in the elementary body of molluscum contagiosum is present in all the pox viruses we have examined. These include Shope fibroma and myxoma, vaccinia, small pox, canary pox, rabbit pox, cow pox, ectromelia and monkey pox.

Significance: The structure, composition and function of this granule may be of fundamental importance and its occurrence may not be limited to the pox viruses. Histochemical methods on tissue sections have been disappointing. However, these will be continued and an attempt will be made to weigh the granule by electron optical methods and isolate it by differential centrifugation so that chemical and biological experiments can be performed to discover its composition and function.

2. Shope fibroma: Electron microscopic structure of associated lamellae.

The striated lamellae associated with Shope fibroma virus infection are made up of disc shaped units 10 nm in diameter and 4 nm thick. Unstriated lamellae are made up of oval units of varying size not clearly resolved but in the order of 7 nm in diameter. The lamellae on cross section are separated from one another by a space containing an irregular dense line. Similar lamellae have been seen in fowl pox of turkey origin, swine pox, ectromelia, rabbit myxoma and canary pox.

Significance: The unit of structure in the lamellae is approaching what might be a basic unit.

Proposed Course: Pictures published of P-fluorophenylalanine treated herpesvirus cultures show structures similar to the striated lamellae. This observation will be followed up.

3. An oyster herpesvirus: This has been found and described. It is the first virus to be found in a bivalve.

Significance: Herpesvirus has been linked to Burkitt's lymphoma and leukemia. Oysters are eaten raw and the significance of this oyster disease to man should be explored. An oyster vesicular cell tumor has been examined by electron microscopy. Particles only suggestive of virus have been found.

Proposed Course: The work on herpesvirus will be published. Vesicular tumors, resembling a lymphoma, have been found in two more species of bivalve. These will be examined with the electron microscope. Further work will be continued by Austin Farley pursuant to a Ph.D. Collaboration with Dr. Dawe in his work in comparative pathology of tumors will continue.

Publications:

1. Banfield, W.G.: Hamster lymphoma, TM. In Lingeman, C.H., and Garner, F.M. (Eds.): Comparative Morphology of Hematopoietic Neoplasms. National Cancer Institute Monograph No. 32, U.S. Dept. of Health, Education and Welfare, Public Health Service, Wash., D.C., U.S. Gov't. Print. Off., 1969, pp. 335-336.

2. Banfield, W.G.: Automation in tissue processing for electron microscopy. In Parsons, D.F. (Ed.): Some Biological Techniques in Electron Microscopy. New York, New York, Academic Press, 1970, pp. 165-174.

3. Ohmori, M., and Banfield, W.G.: Shope fibroma and rabbit myxoma factories. Electron microscopic observations. J. Electron Microscopy 19: No. 4, 1971.

Serial No. NCI 504b
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An Investigation of the Collagenous Connective Tissues and Elastin

Previous Serial Number: Same

Principal Investigator: William G. Banfield, M.D.

Other Investigators: C.K. Lee, L. Sokoloff, A.S. Dekaban, G.R. Martin, W.C. Roberts, and L.M. Buja

Cooperating Units: NIAMD, Lab. Exp. Path., Section on Rheumatic Diseases; NINDB, Surg. Neurology Branch, Section on Child Neurology; NIDR, Lab. of Biochem., Connective Tissue Section; NHI, Cardiology Branch, Section on Pathology

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: To elucidate the normal and abnormal structure of elastin and collagen and their associated cellular and intercellular components.

Major Findings: "Spiney" collagen was found in large amounts in endocardial fibroelastosis associated with rheumatic fever and one case of Hurler's syndrome. Fibrous long spacing (FLS) collagen was also present. These forms of collagen were present in controls consisting of other endocardial fibroelastosis and normal hearts, but were rare. They occurred about vessels in the myocardium but appeared more widely distributed in rheumatic fever.

Significance: "Spiney" collagen and fibrous long spacing collagen has been described in the heart for the first time. It probably represents a collagenous protein polysaccharide complex which forms in abnormal amounts in certain diseased states.

It is of significance that they were present as FLS fibrils in some neoplasms. These include acoustic neurinomas, astrocytomas, peripheral nerve lesions of von Recklinghausen's disease, lymph nodes of Hodgkin's disease,

mouse hemangiosarcomas produced by Moloney sarcoma virus, and canine mastocytomas. "Spiney" collagen has been seen in medullary carcinoma of the human thyroid. In these situations they may reflect a disturbance in the collagen and mucoprotein metabolism, which, if understood, might lead to control of the stroma and possibly the disease.

Proposed Course: To this end we have been working with Dr. Leon Sokoloff, NIAMD, to correlate morphology and metabolism of fibroblasts and chondrocytes in tissue culture.

We propose to continue collaboration with Dr. Sokoloff, and, if personnel and equipment become available, study fibroblasts and chondrocytes from malignant, premalignant and other diseased states correlating structure and metabolism.

Dr. Edward Miller and Dr. George Martin, NIDR, have identified by chemical methods three distinct types of collagen, one found only in cartilage, one in bone and skin and the third in the fetus. These methods should be applied to the stroma of tumors and to connective tissue neoplasms and to the collagen made in tissue culture by fibroblasts taken from tumors. The work would be aimed at both diagnosis and treatment, the latter through control of the stromal connective tissue cell. It will be incorporated into the project already started for which a good base has already been laid.

Dr. Don L. Layman now in NIDR is trained in the chemical techniques required. He also is learning tissue culture and is interested in the project as outlined.

Serial No. NCI 505
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of Potential Pulmonary Carcinogens, Asbestos, Tobacco, Silicates and Heavy Metals and their Relation to the Physiological Status of the Lung

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton, M.D.

Other Investigators: Miss Eliza Miller, Mrs. Connie Wrench, and Miss Betty Sanders and staff

Cooperating Units: Johns-Manville Research and Engineering Center, Manville, New Jersey; Owens-Corning Fiberglass Corp., Toledo, Ohio; Dr. Fred Bock, Roswell Park Memorial Center for Cancer Research; Mel-Labs Inc., Contract NIH 70-2295

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: Work has continued on the two major projects, namely, the elucidation of the mechanisms of asbestos carcinogenesis (which perhaps should be renamed fibrous mineral carcinogenesis) and attempts to develop a satisfactory laboratory model for pulmonary carcinogenesis with tobacco products. Each of these projects are dealt with separately.

Major Findings: Attempts to induce pulmonary carcinoma with tobacco products.

Based on a detailed examination of the morphological response of the lung to chemical carcinogens in beeswax pellets (see previous reports), we have begun a series of experiments to learn whether or not condensates of tobacco smoke alone or in combination with asbestos or glass incorporated in beeswax pellets will induce bronchogenic carcinoma. Findings thus far indicate that the initial response noted with chemical carcinogens, namely development of metaplastic keratinizing squamous epithelium consistently occurs with tobacco condensates but is absent with asbestos or glass. Very preliminary findings suggest that the combination of asbestos and tobacco results in early invasive epithelial lesions. Whether these will evolve into

overt squamous carcinomas remains to be seen. Current experiments are tabulated below. Considerable time has been devoted to quantitative assessment and preparation of the samples and in attempts to separate asbestos and glass into definable size ranges that are comparable with each other as adequate controls. (See Table 1).

Major Findings: Studies of carcinogenicity of asbestos and other fibrous minerals for the pleura of the rat lead to the following conclusions:

1. In contrast to subcutaneous application, crocidolite, amosite, and chrysotile readily induce sarcomas of the pleura, and dose response data can be obtained if the animals are observed over a period of more than two years.

2. The earliest neoplasm was detected at 55 weeks. The mean time of detection with highest dose of conventionally milled South African crocidolite (40 mgm/rat) was 88 weeks.

3. The overall tumor yield employing this type of asbestos was (40-mgm-61%), (20-mgm-48%), (10-mgm-41%), (2-mgm-22%), (1-mgm-4%).

4. Despite an apparent striking difference in the incidence of neoplasms in man resulting from different types of asbestos, no significant difference in carcinogenic response was detected in the rat when various types of asbestos were applied directly to the pleura. The low incidence of mesotheliomas related to chrysotile exposure in man is probably related to failure to reach the pleura rather than to a lesser carcinogenic potential.

5. The popular assumption that contaminating oils or metals from the milling of asbestos may be responsible for asbestos carcinogenicity does not seem valid, since virgin ore milled with the ore itself as the only milling media is as carcinogenic as mine-milled asbestos. Further, previous studies by other investigators have shown that the natural and contaminating mill oils are not significantly carcinogenic and we have shown that the pulverized steel of the type used in the mill and micronized nickel (the most likely metallic carcinogen in the mill) are non-carcinogenic in the system employed.

6. Carcinogenicity is significantly reduced when asbestos is pulverized to a non-fibrous form, indicating that some factor in the physical structure of asbestos is critical to its carcinogenic properties.

7. Fibrous glass of four types that are used commercially (ranging in diameters of 6 to 25 microns, approximately 10X that of commercial asbestos) were not carcinogenic as intact samples. However, when large fibrous glass was pulverized to fibrous particles in the range of asbestos or when exceptionally fine fibrous glass of diameters of $< 3\mu$ was tested an appreciable number of sarcomas developed.

8. A small but significant number of sarcomas comparable to that of pulverized asbestos or glass developed when micronized silica was tested. This suggests that the persistence of a durable irritant may be a factor in carcinogenicity.

Proposed Course: Further experiments into the mechanisms of action of asbestos as a carcinogen are in progress. Electron microscopic analysis of early asbestos induced fibrogenesis and oncogenesis are also being carried out. The unique carcinoma-in-situ like changes in the bronchiectatic lesions of rats are also being studied by electron microscopy with the aim sometime this year of comparing these changes with those that occur in the conversion of respiratory epithelium to keratinizing squamous epithelium as the first step in pulmonary carcinogenesis.

A major effort this year will be directed to confirming our evidence that suggests that the carcinogenicity of asbestos and of glass may relate to certain simple structural qualities in size and shape linked with durability. The importance of fibrous structure is now being studied by investigating the carcinogenicity of other fibrillar minerals such as antigorite, aluminum oxide whiskers, tungsten carbide and various forms of glass and iron. Finding precisely which structural qualities are important in materials used by man is a critical but difficult problem since most materials are mixtures of wide size range and are difficult to separate. We hope to confirm our observations by testing separated fractions of carcinogenic asbestoses, by assessing quantitatively the structural spectrum of the material we have already used, and by testing non-asbestiform fibers in narrow ranges of size comparable to that of different forms of asbestos. The following experiments are currently underway. (See Table 2).

Publications:

1. Stanton, M.F., and Wrench, C.: Factors affecting the development of pleural neoplasms experimentally induced with asbestos and related materials. Talk given at the Symposium on Tissue Response to Asbestos, University of Wales, Cardiff, April 1970.

2. Wrench, C.: Polymerized glutaraldehyde in the fixation of rat lung tissue. In Arceneaux, C.J. (Ed.): 28th Annual Proceedings EMSA. Houston, Texas, 1970.

TABLE 1. CURRENT PELLET EXPERIMENTS

# Rats Surviving Surgery	# Rats Alive End March, 1971	Dates Injected	Dose, Pellet Size and Content
44	37	1/23 - 2/6/70	0.05 ml beeswax tricap. 1:1
10	10	3/16 - 3/17/70	2 x 0.05 ml beeswax tricap. 1:1
50	46	5/12 - 6/5/70	0.1 ml beeswax tricap. 1:1
105	88	2/17 - 3/11/70	0.05 ml crude tobacco + beeswax 1:1
5	5	3/2 - 3/4/70	2 x 0.05 ml crude tobacco + beeswax 1:1
100	94	3/8 - 4/14/70	0.05 ml heptane soluble tobacco + beeswax 1:1
50	45	4/24 - 5/7/70	0.05 ml heptane sol. tobacco + beeswax 1:3
51	48	5/14 - 5/28/70	0.1 ml heptane sol. tobacco + beeswax 1:1
50	42	6/11 - 6/17/70	0.1 ml heptane sol. tobacco + beeswax 3:1
32	32	12/21/70 - 1/7/71	0.05 - 0.08 ml pure tobacco tar heptane soluble
30	28	12/15 - 12/17/70	0.08 ml asbestos + tricap. (200 mgs / 3 ml)

2051

TABLE 1. CURRENT PELLET EXPERIMENTS (Cont'd)

# Rats Surviving Surgery	# Rats Alive End March, 1971	Dates Injected	Dose, Pellet Size and Content
30	30	12/8 - 12/14/70	0.08 ml asbestos + heptane sol. tobacco (200 mgs/3 ml)
26	25	12/2 - 12/3/70	0.01 ml fiberglass + tricap. (100 mgs / ml)
15	12	12/1/70	0.1 ml fiberglass + heptane (100 mgs/ml)
30	30	11/20 - 11/24/70	0.1 ml fiberglass + tobacco - heptane sol. (100 mgs/ml)
30	30	2/12/71 - 2/22/71	0.1 ml asbestos + crude tobacco + tricap. 240 mgs/ml tobacco tricap. 2:3
30	30	2/25/71 - 3/5/71	0.1 ml fiberglass + crude tobacco (± 100 mgs/ml tobacco + tricap. 2:3 ±)
28	28	3/11/71 - 3/12/71	0.1 ml anthracene + beeswax tricap. 1:1 40 mgs/ml
716	660		

GRAND TOTAL

TABLE 2. ASBESTOS AND FIBERGLASS

Experimental Treatment	Dose	Date of Surgery	# Surviving Surgery	# Surviving 4/1/71	# Dead or Killed
1. Whole fiber - Owens-Corning Uncoated Fine Fiberglass	40 mg	4/14/70	30	25	5
2. Unopened Fiber - Cape Blue Ore	40 mg	5/27/70 - 6/1/70	30	27	3
3. Johns-Manville 6D20 Chrysotile	40 mg	7/21-22/70	30	30	0
4. Johns-Manville 6D20 Chrysotile	20 mg	7/31/70	30	22	8
5. Johns-Manville 6D20 Chrysotile	10 mg	8/2/70	30	26	4
6. Johns-Manville 6D20 Chrysotile	5 mg	8/3-4/70	30	29	1
7. Johns-Manville Glass Fibers 5μ X 1μ	40 mg	8/31/70	30	28	2
8. Johns-Manville Glass Fibers 5μ X 3μ	40 mg	9/6/70	30	30	0
9. Johns-Manville Glass Fibers 10μ X 1μ	40 mg	9/13/70	30	29	1
10. Johns-Manville Glass Fibers 10μ X 3μ	40 mg	9/19/70	30	30	0
11. Johns-Manville 6D20 Chrysotile Pulverized 10 Minutes	40 mg	11/23/70	30	28	2
12. Johns-Manville Code 106 Fiberglass	40 mg	12/1/70	30	26	4

2053

TABLE 2. ASBESTOS AND FIBERGLASS (Cont'd)

Experimental Treatment	Dose	Date of Surgery	# Surviving Surgery	# Surviving 4/1/71	# Dead or Killed
12. Johns-Manville Code 106 Fiberglass	40 mg	12/1/70	30	26	4
13. Owens-Corning Sheared Uncoated Fine Fiberglass	80 mg	12/22-23/70	30	29	1
14. Owens-Corning Sheared Uncoated Fine Fiberglass	40 mg	12/29-30/70	30	30	0
15. Owens-Corning Sheared Uncoated Fine Fiberglass	160 mg	1/15/71	30	30	0
16. UICC Crocidolite Pulverized 10 Minutes	40 mg	3/17/71	30	30	0
		TOTALS 4/1/71	480	449	31

TABLE 2. ASBESTOS AND NON-GLASS FIBERS

Experimental Treatment	Dose	Date of Surgery	# Surviving Surgery	# Surviving 4/1/71	# Dead or Killed
1. Antigorite - Pulverized 1 1/2 Minutes	40 mg	7/6-13/70	30	28	2
2. Aluminum Oxide Whiskers	40 mg	9/29-30/70	30	28	2
3. Aluminum Oxide Whiskers	20 mg	10/12/70	30	30	0
4. Aluminum Oxide Whiskers	10 mg	10/19/70	30	26	4
5. Aluminum Oxide Whiskers	5 mg	10/20/70	30	28	2
6. Linde Alumina 0.3μ	40 mg	12/18/70	30	30	0
	GRAND TOTAL 4/1/71		660	619	41

Serial No. NCI 514
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Induced and Spontaneous Neoplasms in Laboratory Animals. Factors that Influence their Incidence and the Chemical Changes Induced by the Neoplasms

Previous Serial Number: Same

Principal Investigator: Ambadas S. Mulay, Ph.D.

Other Investigators: R.W. O'Gara, M.D. (Deceased)

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1
Professional: 1/2
Other: 1/4

Project Description:

A. Objectives: To establish a dose response curve for carcinogen N,N-dimethyl-p-(m-tolylazo)-aniline (me-DAB) when fed in conjunction with complete diet of powdered Purina laboratory chow.

Methods Employed: Accurately weighed carcinogen was incorporated either in semisynthetic or in powdered Purina laboratory chow and fed to the animals for 4 months and experiment was terminated 2 months later. Male rats of Osborne-Mendel, NIH Black, and Marshall 520 strain and females of Osborne-Mendel strains were used in these experiments. Animals were examined every day and some were killed at regular intervals for biochemical and histological studies.

Major Findings: It takes higher concentration of me-DAB to induce hepatomas in rats on Purina laboratory chow diet than those on semisynthetic diet. 0.06% me-DAB gives high incidence of hepatomas in rats on semisynthetic diet. On Purina laboratory chow diet, it takes 0.09% me-DAB for male and 0.12% me-DAB for female Osborne-Mendel rats to get similar incidence of hepatomas. 0.06% me-DAB gives about 20% cholangiofibrosis in livers of male and no significant change in those of female rats. 0.09% me-DAB gives some cholangiofibrosis and one (10%) hyperplastic nodule in female rats. Incidence and histology was similar in all three strains of rats--Osborne-Mendel, NIH Black, and Marshall 520. Three of the hepatomas induced in NIH Black rats were successfully propagated through 8 transplant generations.

Significance: Dose response curves for me-DAB fed in Purina laboratory chow to male and female Osborne-Mendel rats have been established. These curves could be used as base lines for the study of the effects of other agents including carcinogens on me-DAB carcinogenesis.

Proposed Course: No further experimental work is planned on this project. A paper dealing with the dose response curve for 3'-Me-DAB is submitted for publication.

B. Objectives: Induction of glandular stomach lesions in guinea pigs.

Methods Employed: A group of male guinea pigs of Hartley strain are given by stomach tube, a suspension of nitrosomethyl urethane 3 times a week; and another group receives the suspension, by intraperitoneal route once a week. Animals are examined for tumors and other changes at weekly intervals.

Major Findings: Experiments are in their preliminary stage. No findings to report at this time.

Significance: A reliable method of producing high incidence of gastric carcinoma will give us a tool to study the chemistry of the lesion and may be useful in determining relative effectiveness of various chemotherapeutic agents.

Proposed Course: The work will continue in the next fiscal year.

C. Objectives: To study the effect of N,nitrosodiethylamine (DENA) a carcinogen, on N,N-dimethyl-p-phenylazoaniline (DAB) hepatocarcinogenesis in the rat.

Methods Employed: Rats received 5 mg % DENA in drinking water for 4 months. Some of these animals received a subcarcinogenic dose of DAB (0.12%) in Purina laboratory chow diet for the same period. Another group of rats received only 0.12% DAB in their chow diet. The experiments were terminated 2 months later and the animals killed for biochemical and histological studies.

Major Findings: Preliminary results show that DENA alone induces cholangiofibrosis and hemorrhagic lesions of the liver in high percentages of rats that survive the experimental period. The animals receiving combination treatment of DENA and DAB seem to show more hepatoma nodules and DAB alone shows very little change in liver histology.

Significance: Interaction of one carcinogen on the carcinogenic action of another may give some clues to their mode of action.

Proposed Course: The work of tabulating the data, describing the lesions induced, and evaluating the results is in progress. No further experimental work is planned on this project.

D. Objectives: Induction of glandular stomach lesions in rats.

Methods Employed: Male rats of Osborne-Mendel strain and male and female rats of Wistar strain have been started on N-nitrosoguanidine in drinking water.

Major Findings: Tumors have been observed in the glandular stomach of three Osborne-Mendel and one Wistar rat. Their histology is under study.

Significance: Induction of glandular stomach tumors could lead to development of a transplantable stomach tumor. This could prove to be an interesting experimental tool.

Proposed Course: Work will continue in the next fiscal year.

E. Objectives: Induction of lung tumors in rats.

Methods Employed: Osborne-Mendel rats are tube fed twice a week with freshly prepared solution of 4-nitroquinoline-1-oxide in 10% dimethyl sulfoxide. The rats will be examined at regular intervals for signs of tumors.

Major Findings: Experiments have just started. No findings thus far.

Significance: Transplantable lung tumors could be used as an interesting experimental tool.

Proposed Course: Work will be continued next year.

Publications:

1. Mulay, A.S., and O'Gara, R.W.: Effect of diet on N,N-Dimethyl-p-(m-tolylazo)aniline carcinogenesis in rats. Proc. Soc. Exp. Biol. Med., In Press.

Serial No. NCI 516c
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Viral Carcinogenesis. The Murine Sarcomagenic Viruses: Problems of Immunity, Resistance, and Pathogenesis and their Relation to Cell Structure

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton, M.D.

Other Investigators: Miss E. Miller, Mrs. C. Wrench, and Miss Betty Sanders and staff

Cooperating Units: Dr. Lloyd Law and staff (NCI 3200)

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: Studies of histopathological and fine structure alterations related to the pathogenesis of disease induced by a murine sarcoma virus.

Major Findings: Experience gained from examining neoplasms induced by this unit and by others strongly suggests that all of the neoplasms may have a common cell of origin. If this is the case, the most likely candidate would be a primitive cell of the reticuloendothelial-histiocytic series. Derivation of all of the neoplasms from cells closely related to lymphoreticular neoplasms would of course be most significant to the phenomenon of C-type virus oncogenesis.

As previously reported, the disease induced by this virus in both rats and mice is initially an inflammatory granuloma in which atypical cells arise. This response occurs in many tissues although definite tissue-selectivity occurs. Ultimately a small percentage of the lesions evolve into simple neoplasms. The unique reactive response to either the atypical cells or the murine sarcoma virus and its relation to leukemogenesis and such neoplasms in man (for example, Kaposi's disease, Hodgkin's disease and the reticuloendotheliosis) that show similar reactive components make this disease a particularly critical one in studies of viral carcinogenesis. Further studies in viral oncogenesis have centered on characterization of cell structure and

cell alterations induced by infection with so-called "murine sarcoma virus" by electron microscopy.

Proposed Course: The current problem is one of seeking ways of clearly and unequivocally establishing the progenitor cell type of the neoplasms. No simple means is at hand and investigations may be deferred for some time unless a reasonable approach to this problem can be devised. Nevertheless, the electron microscopy, histology and distributional analysis of lesions from several hundred rats are being analyzed. Of particular interest is our effort to compare changes induced in the tumors of osseous and neurogenic tissues with those in the transplantable MSB-1. Analysis of tissue morphology by a variety of means will probably occupy the better part of the year.

Publications:

1. Stanton, M.F., Ting, R.C., and Miller, E.: Cytologic Specificity of Response to Moloney Murine Sarcoma Virus as Evident in a Transplantable Epithelioid Neoplasm of the Rat. J. Nat. Cancer Inst. 45: 195-209, 1970.

Serial No. NCI 519
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Induction of Neoplasms, Selection of Transplantable Strains of Tumors, and Study of Tumor and Host Chemistry

Previous Serial Number: Same

Principal Investigator: Ambadas S. Mulay, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1
Professional: 1/2
Other: 1/4

Project Description:

A. Objectives: To induce glandular stomach tumors in rats and to establish a transplantable strain of these tumors.

Methods Employed: A group of male rats of Osborne-Mendel and Buffalo strain are fed a semisynthetic diet deficient in vitamin "A" to which carcinogen, 2,7-Diacetamidofluorene is added suspended in corn oil. Another group of rats are fed the same diet except for the addition of an irritant to it - celite. Diets are thoroughly mixed with a mechanical mixer and stored at $0^{o}C$. Fresh diet is prepared every other week. Animals are examined every week for their general health and possible tumors.

Major Findings: The experiments have been going on for only three months. There are no findings to report at this time.

Significance: This experimental study may throw some light on the mechanism of induction of glandular stomach tumors. Transplantable tumors may serve as a tool in further study of this neoplasm.

Proposed Course: The study is to continue through the next year.

B. Objectives: To induce kidney tumors in rats developing a transplantable kidney tumor.

Methods Employed: Rats, Osborne-Mendel, Fischer, and Marshall 520 are fed carcinogen N-nitrosodimethylamine in Purina laboratory chow for various times and animals observed for signs of tumor induction and transplanting the induced tumors to establish transplant lines.

Major Findings: Kidney tumors have been induced in most of the experimental rats of Osborne-Mendel and Fischer strain. A transplantable kidney tumor has been established in Osborne-Mendel strain of rats, and it is in its 10th transplant generation.

Significance: This transplantable kidney tumor could be a useful experimental tool.

Proposed Course: The results will be evaluated, documented and prepared for publication.

C. Objectives: To induce glandular stomach tumors in rats and to establish a transplantable strain of these tumors.

Methods Employed: (a) Synthesize the chemical 4-hydroxyaminoquinoline-1-oxide. Feed the compound in food, in water or by stomach tube to Osborne-Mendel, Fischer, Marshall and Wistar rats. (b) Similarly tube feed freshly prepared N,nitrosoguanidine solution to rats twice a week. Examine the animals for tumors at regular intervals. Transplant the primary tumors as they become available and try to establish a transplantable line.

Major Findings: The first batch of 4-hydroxyaminoquinoline-1-oxide has been synthesized and first feeding experiments just started. It is too early for any findings.

Significance: Transplantable glandular stomach tumor could be an interesting experimental tool for studying the characteristics of this tumor.

Proposed Course: The study is to continue through the next year.

Serial No. NCI 521a
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Investigations of Mechanisms of Tumor Induction by Polyoma Virus in Mice

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe, M.D.

Other Investigators: Dr. Jacqueline Whang-Peng, Dr. William G. Banfield, and Mr. Willie D. Morgan

Cooperating Units: Medicine Branch, NCI

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives: This continuing project is designed to clarify the conditions necessary for and the cellular activities leading to tumor development in mouse salivary glands infected with polyoma virus. Previously published works from this project have shown the following:

1. In PV-infected organ cultures of salivary glands, morphologic transformation occurs, closely resembling tumor development in vivo. When such transformed cultures are transplanted to mice, typical salivary gland tumors appear at the site of transplant.

2. In the organ culture system, the visible sequence of events is: (a) Lytic infection of mesenchyme; (b) lytic infection of epithelium; (c) emergence of transformed cells, some of which undergo viral lysis while others proliferate.

3. At very early stages of embryonic development, salivary gland or pre-salivary tissue does not undergo neoplastic transformation subsequent to PV infection.

4. When the epithelial and mesenchymal components of embryonic salivary glands are isolated from each other by trypsin dissection neither component is able to give rise to a neoplasm after infection with PV.

5. Recombination of salivary epithelium with salivary mesenchyme restores the capacity for tumor development to occur. Recombination of salivary epithelium with tooth bud mesenchyme, or of tooth bud epithelium with salivary gland mesenchyme, does not restore the capacity for tumor development.

6. PV-induced tumor tissue can substitute for normal salivary gland mesenchyme in supporting morphogenesis in vivo. In vitro, the tumor tissue has so far been observed to support growth, but not morphogenesis of salivary epithelium.

7. Epithelial origin of the PV-induced salivary gland tumors has been demonstrated (see ref. to publication). This was done by reassembling glands from T6T6 chromosome-marked epithelium and unmarked mesenchyme, then determining the presence or absence of the marker chromosomes in tumors induced by PV in the reassemblies.

Specific objectives are as follows:

1. To determine the degree of specificity of the growth and/or development-supporting effects manifested by the salivary gland tumors.

2. To compare, using light and electron microscopy, the development of salivary epithelium when supported (a) by normal mesenchyme, and (b) by PV-induced tumor.

3. To determine whether differences may be found in the interaction between normal salivary epithelium and normal mesenchyme on the one hand, as compared with the interaction between normal salivary epithelium and PV-induced tumor on the other. This is being done by Mr. Morgan, by making time-lapse phase-contrast motion pictures of the interacting cell groups in tissue cultures.

4. To determine whether the growth and/or development-supporting effects are effective across porous membranes that prevent cytoplasmic contact between interacting cells. This project will be extended in the coming year to include testing for trans-filter tumor-inducing activity of salivary tumor cells placed opposite normal salivary epithelium, with or without PV virus.

Methods Employed: In part 1 above, isolated epithelium from various organ rudiments is placed within a small tumor explant and the ensemble is transferred subcutaneously to a syngeneic newborn mouse. After 12 days, the transplant is removed, serially sectioned, and examined to determine the degree of differentiation that has taken place in the normal embryonic epithelium that was placed in the tumor. Special stains are performed on some sections to determine whether basement incumbrances have been formed or whether enzymes are present in the epithelium.

In part 2 above, the electron microscope is used to study the fine structural aspects of tumor cells interacting with salivary epithelium as the latter undergoes development in the tumors. Specifically, we are interested in knowing whether basement membranes are formed between tumor cells and normal epithelium, whether zymogen granules can be identified in the epithelium, whether virus is present in either tumor or normal cells, and what is the nature of contacts or junctions between (a) tumor cells and tumor cells; (b) tumor cells and normal epithelium; (c) normal epithelium and normal epithelium; (d) normal epithelium and normal mesenchyme.

In the time-lapse study, motion pictures of interactions between the same 4 combinations listed above will be studied. The technical aspects of this work will be done by Mr. Morgan, using a tissue culture chamber which he adapted particularly for this type of work.

In the transfilter studies, tumor or tumor extract is placed on one side of a Millipore filter, with the epithelium on the other. The epithelium is observed for growth and differentiation. In preliminary experiments, epithelium is placed directly in contact with tumor tissue, or is immersed directly in the extract of tumor tissue. Evaluation of results will be based on direct observations of living cultures and on microscopic examination of serial sections.

Major Findings: 1. The experiments demonstrating epithelial origin of the PV-induced salivary tumors have now been published, and will not be described again here.

2. In the trans-filter experiments, the significant findings are:

(a) PV-induced salivary tumor cells support growth of normal salivary gland epithelium across the membrane.

(b) PV-induced fibrosarcoma cells likewise support growth of normal salivary gland epithelium across the membrane.

(c) Spontaneous mammary tumor agent containing mammary tumor cells fail to support growth of normal salivary gland epithelium across the membrane. This observation is still considered preliminary, as it rests on a relatively small number of negative results.

(d) Normal salivary gland mesenchyme retains its ability to support salivary epithelial growth across the membrane for at least 6 days after being isolated from the normal rudiment, in tissue culture. This observation will make control experiments technically easier, since it will allow time for mesenchyme to become attached to the membrane before inverting the chamber and adding epithelium, which depends on gravity for establishing initial contact with the membrane.

(e) A technical advance has been made by using trypsin-versene dispersal of tumor cells before attaching them to one side of the membranes. This allows more uniform reproducibility of culture conditions.

Significance: Any factor that enables or forces cells to proliferate under conditions otherwise not compatible with proliferation, is of interest in cancer research. Since the salivary tumor cells appear to be functioning in much the same manner as normal salivary gland mesenchyme, so far as growth and development are concerned, it seems likely that we are dealing with the same phenomena involved in normal growth and differentiation. The point of main interest is that the salivary gland tumor cells are of epithelial origin, and since they are no longer dependent on mesenchyme for growth support, it appears that they have acquired the ability to produce their own growth-supporting substances. This suggests the possibility that the polyoma virus induces neoplasia by bringing about derepression of some gene activity in epithelium, whereas normally this derepression would occur only in mesenchyme, which serves as a growth regulator on epithelium. If this concept is correct, then the same type of derepression must occur in subcutaneous fibroblasts under the influence of PV. Until more work concerning the specificity of various tumors for production of the growth response is completed, it would be premature to draw conclusions.

Proposed Course: In the coming year, work will be directed along 2 lines:

1. To observe the effects of PV on normal salivary epithelium supported by salivary gland tumor cells and by fibrosarcoma tumor cells across the millipore membrane. Controls will be normal salivary epithelium supported by normal mesenchyme. The normal epithelium will come from T6T6 mice, so that tumors originating in it can be positively identified. The identification of neoplastic transformation will be based on the appearance of T6T6-marked tumors in mice receiving the presumptively transformed epithelium. Additional controls will consist of epithelial cultures not infected with PV, but also supported trans-filter by salivary gland tumor cells. It is possible that these controls may reveal an abnormal, neoplastic "inducer" in the tumors.

2. Extension of the study to include mammary gland. The ability of salivary tumor tissue to support growth of mammary epithelium will be tested *in vivo* and *in vitro*. Also, the ability of mammary tumor cells to support growth of normal mammary epithelium will be tested. We hope to be assisted in this by Dr. Teruyo Sakakura, who will work with the group for one year as a Visiting Associate.

Publications:

1. Dawe, C.J., Whang-Peng, J., Morgan, W.D., Hearon, E.C., and Knutsen, T.: Epithelial origin of salivary tumors in mice: Evidence based on chromosome-marked cells. *Science* 171: 394-397, 1971.

Serial No. NCI 538
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Study of Phylogenetic Aspects of Neoplasia

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe, M.D.

Other Investigators: Dr. John C. Harshbarger, Dr. William G. Banfield, Dr. David Small, Dr. Costan W. Berard, Dr. Maire F. Mulcahy, and others

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives: The continuing major objective of this project is to perform comparative studies of neoplasia in invertebrate and lower vertebrate animals, in order to gain a comprehensive view of the biology of neoplasia throughout the various animal phyla. This is done by collecting neoplasms and studying their morphology and natural history. Laboratory studies concerned with induction and transplantation of neoplasms in lower animals are also conducted. Phenomena such as regeneration, dedifferentiation, autotomy, molting, metamorphosis, graft rejection, and so on are studied in relation to the biology of neoplastic disease. The project also includes collection of literature concerned with neoplasms of invertebrate animals.

Major Findings: 1. A morphologic study of epizootic lymphoid neoplasms of northern pike in Ireland has been completed, in a collaboration with Drs. Maire F. Mulcahy and G. Winqvist. By light microscopy and cytochemical methods, it was found that the type cell of these neoplasms is identical from case to case, and that it is an undifferentiated (blastoid) stem cell, possibly an immunoblast. The nucleus has the features of a highly immature cell, and the cytoplasm lacks specific granules of any type associated with the various lines of normal leukocytes. The cytoplasm is strongly basophilic and pyroninophilic, contains a few droplets of neutral fat, and is strikingly distinguished by strong acid phosphatase reaction in the area occupied by and adjacent to the Golgi apparatus. This same area is weakly positive with the periodic acid-Schiff reaction. The cells are non-phagocytic.

By electron microscopy, the cells were seen to contain many mitochondria with tubular cristae, scant endoplasmic reticulum with abundant free ribosomes, and occasional multivincular bodies sometimes containing crystalline lattice arrays within them. In a few cells the endoplasmic reticulum was arranged in 2 or 3 concentric layers parallel to the plasma membrane, suggestive of the pattern characteristic of immature plasma cells. No intra- or extracellular virus particles were found.

2. Tissue culture studies of a chondrosarcoma in a corn snake are in progress. This neoplasm was obtained from Dr. David Small, presently at the Johns Hopkins School of Medicine's Animal Care Laboratory. The snake was for many years a specimen in the Baltimore Zoo.

The chondrosarcoma cells have been growing quite well both in gelatin sponge cultures and directly on glass in Leighton tubes for 4 months. Cells in the sponge cultures sometimes contain eosinophilic cytoplasmic inclusion bodies which probably correspond to crystalline arrays of dense spherical bodies found with the electron microscope by Dr. Banfield. These are not virus particles, but are probably mineral deposits related to the calcifying tendency shown by the tumor both in vivo and in sponge cultures.

3. In conjunction with the activities of the UICC's Committee on Comparative Oncology, a study of naturally-occurring liver tumors in the bottom-feeding fish, Catastomus commersonii (white sucker) is being re-initiated. In an earlier (published) study with Dr. M.F. Stanton and F. Schwartz, it was found that very large fish of this species taken from Deep Creek Lake, Maryland, frequently bear large cholangiomas or cholangio-carcinomas. The cause is unknown, but 4 possible etiologic factors are under consideration: (a) hydrocarbons released into the water by motor boat exhausts; (b) chlorinated hydrocarbon pesticides contaminating the lake from agricultural and mosquito-control activities; (c) a protozoan parasite (Thelohania sp.) associated with the neoplasms and also present in non-tumor bearing fish; (d) rotenone, a polycyclic hydrocarbon with structure similar to methylcholanthrene, which is used in Deep Creek Lake by Sports Fisheries biologists in collecting fish population samples.

Subsequent to the Deep Creek Lake survey, one specimen of C. commersonii has been found in a lake on which motor boats are not used and in which rotenone has not been employed. Collections are being continued with assistance from Biologist David Woronecki, of the Maryland Bureau of Sports Fisheries.

As chairman of the UICC Committee on Comparative Oncology, the principal investigator is responsible for the organization of a survey of filter-feeding and bottom-feeding aquatic animals. The object of this survey is to determine whether these aquatic animals may be useful as indicators of environmental carcinogens. The project was fully described in last year's report and in the UICC Cancer Bulletin cited in the references in this report.

4. A Workshop on the Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms has been organized under co-chairmanship of the principal investigator and Dr. Costan W. Berard. This workshop will be held at NIH on May 10-14, 1971, and will bring together investigators of blood cell neoplasms in animals ranging from mollusks to man. Histologic study sets of 140 slides have been prepared, and 30 sets of these will become a part of the tumor type collection of NCI's Registry of Experimental Cancers. An Atlas based on this material will be prepared following the workshop. This project is primarily educational rather than investigative. It is expected nevertheless to act as a stimulus to extended investigations, particularly in the area of cold-blooded vertebrate and invertebrate animals.

Significance: 1. Lymphoma of northern pike. The strict cytologic uniformity among the cases is consistent with 3 possibilities: (a) that the hematopoietic stem cell in this species is particularly prone to become neoplastic; (b) that a highly specific agent such as a virus acts on this cell type alone to produce the disease; (c) that the neoplasm is transmitted from fish to fish by cellular inoculation, e.g., through biting. Epizoological evidence on a similar disease in muskellunge presently favors the last possibility, as does the absence of a demonstrable viral agent. More investigation is needed to clarify this question.

The morphological studies have clearly established the properties of the neoplastic cell-type, so that this neoplasm can henceforth be distinguished from other hematopoietic neoplasms of pike, and can serve as a reference standard for comparisons with similar neoplasms in animals and man.

2. The snake chondrosarcoma cells are being cultured chiefly with the aim of seeing whether or not they may be virus producers. A myxosarcoma from a Russell's viper has previously been found (by Zeigel and Clark) to produce C-type virus particles. The chondrosarcoma cells may also prove useful to investigators interested in cartilage matrix formation.

3. The study of hepatic tumors in C. commersonii at present merely adds another bit of information on neoplastic disease in feral aquatic animals. If the etiology can be determined, this animal may be useful for controlled studies of mechanisms of action of biliary carcinogens. The survey of filter-feeding and bottom-feeding animals will hopefully yield dividends in the form of information on environmental carcinogens active also in man.

4. The Workshop on Hematopoietic and Lymphoreticular Neoplasms affords investigators of these neoplasms in animals at various phylogenetic levels an opportunity to see the relevance of their work to research on leukemia in man. Fundamentally, the leukemias and lymphomas are neoplasms of the cellular immune surveillance system, which has a vital function at all levels down to and including protozoa. Reasons must exist to explain why this system is more susceptible to neoplastic transformation in some animals than in others. Epizootics such as those of lymphomas and leukemias in pike, muskellunge, and certain mollusks should be especially useful in attacking this problem, as well as in probing the oncogene theory of neoplasia.

Proposed Course: The collaboration in the pike lymphoma study has been completed, and no extension is contemplated. Other laboratories in Ireland and Canada are continuing epizootiologic, transmission, virologic, tissue culture, and cytogenetic studies.

The survey of cholangiomas in C. commersonii will be continued, with addition of tissue assays in search of possible correlations between tumor occurrence and tissue pesticide content.

The snake chondrosarcoma will be more extensively studied for evidence of virus production and presence of RNA-dependent DNA polymerase.

A year or more may be required for preparation of an Atlas on Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms.

Publications:

1. Mulcahy, M.F., Winqvist, G., and Dawe, C.J.: The neoplastic cell type in lymphoreticular neoplasms of the northern pike, Esox lucius L. Cancer Res. 30: 2712-2717, 1970.

2. Dawe, C.J.: Comparative oncology and environmental carcinogens. UICC Bulletin 8: 2-3, 1970.

3. Dawe, C.J.: Neoplasms of blood cell origin in poikilothermic animals. A status summary. In Dutcher, R.M. (Ed.): Comparative Leukemia Research. Karger, Basel, 1971, pp. 634-637.

Serial No. NCI 579
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Action of Methylazoxymethanol on Rat Mesonephros and Metanephros in Organotypic Culture

Previous Serial Number: None

Principal Investigator: Shozo Takayama, M.D. (Eleanor Roosevelt Fellow)

Other Investigators: Dr. Gert L. Laqueur, Dr. Maria Spatz, and Dr. Clyde J. Dawe

Cooperating Units: None

Man Years (August 1, 1969 - August 1, 1970)

Total:
Professional:
Other:

Project Description:

Objectives: Methylazoxymethanol (MAM) is the active aglycone moiety of the naturally-occurring carcinogen, cycasin. It was shown by Laqueur and collaborators to induce renal neoplasms in adult rats and to induce neoplasms of intestine and kidney in the offspring of female rats exposed to the carcinogen during pregnancy. It therefore can act transplacentally on renal tissues during relatively early stages of renal development. The object of this project is to determine whether MAM induces neoplastic change in embryonic mesonephros and metanephros in organ culture.

Methods Employed: Accurately staged Fischer rat embryos are aseptically removed from the uterus at 13, 14, and 15 days of gestation. Mesonephric and metanephric rudiments are excised and exposed to MAM acetate at several concentrations for 12 or 24 hours, and are then placed in gelatin sponge matrix cultures for periods up to 30 days. Living cultures are observed for toxic effects of the carcinogen, and cultures are subsequently fixed, serially sectioned, and examined histologically for evidence of changes consistent with neoplastic transformation. In other experiments, similar kidney rudiments are exposed to the carcinogen and are then transplanted to newborn syngeneic rats to see if neoplasms arise in the exposed, transplanted organs. Experiments similar to those in organ culture are done with monolayer cultures prepared from embryonic and newborn rat kidney.

Major Findings: Levels of MAM acetate that produce toxic but sublethal effects on the organ rudiments have been established, and the long-term response to these levels is being observed. Thus far morphologic changes consistent with neoplastic transformation have not been observed, but the study is not completed. Insufficient time has elapsed for evaluation of the experiments dealing with transplants of renal tissue briefly exposed in vitro.

Significance: If carcinogenicity of MAM can be demonstrated on rat kidney organ cultures, the method can then be applied to human renal tissue in culture. Changes comparable to those seen in rat tissues would constitute presumptive evidence of carcinogenic action of MAM in man. The method could then be more widely applied as a presumptive test for carcinogens active on human tissues.

Proposed Course: Dr. Takayama's Fellowship is limited to one year, but he plans to continue the work on return to Japan. Animals bearing transplanted, MAM-exposed renal tissue will be maintained at NCI until evidence of tumors appears. If tumors do not appear, the animals will be autopsied at 2 years of age. Additional experiments are under way, to determine whether kidneys of rats exposed to MAM in utero show neoplastic change in organ culture. These will serve as positive controls. Older kidneys will also be tested.

Publications: None to date. Results will be submitted for publication after completion of work.

Serial No. NCI 560
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electron Probe and Scanning Electron Microscope

Previous Serial Number: Same

Principal Investigator: William G. Banfield, M.D.

Other Investigators: Philip Grimley, William G. Hammond, and Biodynamics Research Corporation

Cooperating Units: Biodynamics Research Corporation; Surgery Branch, NCI

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: 1. Learn how and in what areas the electron probe scanning electron microscope and ion probe can be applied to biological research.

2. Work out quantitative methods for analysis of biological specimens.

3. Keep abreast of these instruments and make contributions to their development as applied to biology.

Major Findings: Electron probe analysis for iodine in human thyroid and parathyroid glands, normal and neoplastic. Analysis for iodine in human thyroid and parathyroid tissue, normal and neoplastic was accomplished using the electron probe. Iodine within the limit of detection, 0.04%, was found in the follicular colloid of normal thyroid and thyroid adenomas but was absent in the cells lining the follicles. Paraffin embedding and hematoxylin and eosin staining did not diminish the detectability. With one exception, iodine was not found in the colloid-like material or cells of parathyroid glands. Iodine was not found in parathyroid adenomas, nor in the colloid or cells of thyroid carcinomas, nor in the amyloid or cells of medullary carcinoma of the thyroid.

Significance: The above work was accomplished by using an especially built light transmission stage which allowed for easy localization of the electron beam. It shows that such an approach is feasible. If readily

available, it would open up a wide field of knowledge in the analysis of light microscopically visible lesions and cell inclusions. This would be especially valuable to the pathologist.

Proposed Course: A contract will be worked out with the National Bureau of Standards (NBS) to apply the analytical capability of EMMA (electron probe transmission electron microscope), scanning electron microscope, crew type scanning electron microscope, and ion probe to biological problems. This will take continued improvement in instrumentation, analytical methods (specimen preparation and methods of quantitative analysis) and training of personnel in the operation of the instruments and interpretation of results. These instruments when used for analysis are not merely "another electron microscope". They are, rather, very powerful instruments which are becoming increasingly sensitive in combining structural information with chemical composition. NBS has stated its interest in such a program on pages 17 and 18 of the July 1969-June 1970 Report of the Activities of the NBS Spectrochemical Analysis Section. "Unfortunately this is the most difficult area within the realm of quantitative electron probe microanalysis. In view of the urgency of progress in this field, we hope soon to broadly attack this important subject to which we have already given much attention in the past years."

Publications:

1. Banfield, W.G., Grimley, P.M., Hammond, W.G., Taylor, C.M., deFlorio, B., and Tousimis, A.J.: Electron probe analysis for iodine in human thyroid and parathyroid glands, normal and neoplastic. J. Nat. Cancer Inst. 46: 269-273, 1971.

Serial No. NCI 561
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Collaborative Research and Other Activities

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton, M.D.

Other Investigators: None

Cooperating Units: SVCP, SALES, JNCI

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/2
Other: 0

Project Description: (Other Activities)

Fifty percent of the normal work week is spent as Scientific Editor of JNCI. Ten percent of the normal work week is spent in administrative activities related to contracts with the Special Virus Cancer Program, Comparative Pathology Contract with the Smithsonian Institution, and long-term animal holding facility contract with Melpar, Inc.

Proposed Course: These activities will probably continue. In addition, time will be devoted to training in electron microscopy, preliminary investigations in the study of neoplasia in lower forms of animal life, and preparation of a monograph on bone tumors in mice.

Publications:

1. Harshbarger, J.C., Cantwell, G.E., and Stanton, M.F.: Effects of N-Nitrosodimethylamine on the crayfish, Procambarus clarkii. J. Invert. Path., In Press.

Serial No. NCI 527
1. Laboratory of Pathology
2. Cytopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunocytology and Physiopathology of Cancer

Previous Serial Number: Same

Principal Investigator: Richard A. Malmgren, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1970)

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: 1. To apply the fluorescent antibody techniques and other immunologic methods to the study of tumor protein and antigens. 2. To study the immunologic and other factors involved in carcinogenesis.

Methods Employed: 1. The fluorescent antibody technique has been used to study various antigens associated with neoplastic cells. This requires the preparation of antisera to the appropriate antigen and tagging the antibody with fluorescein isothyocyanate. The antisera thus prepared are applied to the cells under study, either tissue imprints, tissue culture cells or frozen sections and examination of these cells in an ultraviolet microscope. The studies which have been performed during the past year include:

1A. Examination of antiosteosarcoma and liposarcoma antibody in the serum of sarcoma patients, their relatives and associates, and in normal individuals. (In collaboration with Dr. Morton.

1B. Studies of immunologic factors in malignant melanoma (In collaboration with Drs. Morton, Eilber and Wood).

1C. Demonstration of viral particles in human liposarcoma (In collaboration with Dr. Hall and Dr. Morton).

1D. Study of transformation of hamster cells in vivo and in vitro by an adenovirus 4-SV40 hybrid. (In collaboration with Dr. Easton and Dr. Rabson).

2A. Studies of the effect of anti-lymphocyte serum on interferon production. (In collaboration with Dr. Barth and Dr. Friedman).

2B. Effect of anti-lymphocyte serum on the reticuloendothelial system. (In collaboration with Dr. Sheagren, Dr. Barth and Dr. Edelin).

3. Establishment of human foetal intestinal agar culture for growth of virus. (In collaboration with Dr. Dolin, Dr. Blacklow and Dr. Chanock).

4. Malignant lymphomas were produced in mice treated with immunosuppression and antigenic stimulation. (In collaboration with Dr. Krueger and Dr. Berard).

Major Findings: 1A. Serum from patients with osteosarcoma reacted by immunofluorescence in all instances with their own tumors and with osteosarcoma cells from other patients. Serum from their relatives and associates were reactive in 85-91% of the cases while normal blood donor sera reacted only 29% of the time and then at a much lower dilution. The specificity of the reaction was demonstrated by blocking tests. The sarcoma antigen was passed to normal human fibroblast culture from cultures of the sarcoma cells. Transformation of the normal cells was also observed when exposed to the sarcoma culture fluid. Virus particles were seen by electron microscopy in the transformed cells.

1B. Antibody titer to melanoma correlated with the stage of the disease being higher with localized disease than with widespread metastases. The same was true for delayed cutaneous hypersensitivity. Treatment with BCG caused a rise in anti-melanoma antibody titer and temporary tumor repression.

1C. Type C particles were observed by electron microscopy in human liposarcoma. Although not the first report of Type C particles in human tumors the association between this observation and the in vitro transformation of normal cells by cell-free material from these cultures as well as the transfer of antigen to normal cells by cell-free extracts of these cultures strongly points to a viral etiology for these human sarcomas.

2A. Anti-mouse lymphocyte serum was shown to inhibit interferon production in mice exposed to Poly I:C or to Sendi virus.

2B. Antilymphocyte serum was shown to inhibit the phagocytic activity of the R.E. system.

3. Human foetal intestine cells were grown in organ culture in agar and support the growth of virus.

4. The combination of immunosuppression and antigenic stimulation produced malignant lymphomas whereas neither alone did.

Significance: 1A. In considering how to study human tumor antigens with the purpose of immunotherapy or demonstration of a viral etiologic agent one continually returns to the belief that the only approach is the study of the patient's tumor with his own serum. This is the inevitable conclusion because the complexity of any other antigenic system becomes so enormous that it defies unraveling. The demonstration of antisarcoma antibody in the patient's own serum points clearly to an immunologic response on the part of the patient which indicates the tumor to be antigenic. The possible viral nature of this antigen is suggested by similar antibody being demonstrable in other sarcoma patients as well as the sera of their associates. The transference of this antigen by cell free extracts to normal cells and the demonstration by E.M. of virus in these normal cells transformed by the extract suggests a viral agent.

1B. The effect of the extent of the neoplastic disease on the anticancer antibody titer, or visa versa suggests the idea that immunotherapy may be possible. This is augmented by the observed influence of BCG on the disease.

1C. Finding viral particles in human neoplasms along with corroborative immunologic findings suggests the viral etiology of human cancer.

2. Efforts to understand the mechanism of action of antilymphocyte serum on the immune response suggest that a number of components of the immune system are involved. These studies demonstrate that the macrophage behavior is effected by ALS, that interferon formation is effected as is phagocytic activity.

3. The growth of virus in intestine organ cultures makes possible the propogation of virus which have previously been difficult to work with in the laboratory.

4. The importance of immunosuppression and antigenic stimulation as etiologic factors in malignancy is suggested. Immunosuppression as a common theme for carcinogenic agents has been previously suggested by this laboratory and the added dimension of metabolic stimulus to the effector organ is suggested by the observed lymphoma formation when antigenic stimulus is added.

Proposed Course: 1. The use of the fluorescent antibody technique for the study of tumor antigens will be examined. 2. Further studies of the host cell relationship in the metastatic process will be undertaken. 3. Additional studies of the factors, particularly immunologic, involved in carcinogenesis will be carried out. 4. Increased emphasis will be placed upon studies of cellular immunity.

Publications:

1. Sheagren, J.N., Barth, R.F., Edelin, J.B., and Malmgren, R.A.: Anti-lymphocyte serum blockade of the reticuloendothelial system. Amer. Heart J. 81: 144-145, 1971.

2. Hall, W.T., Morton, D.L., and Malmgren, R.A.: Virus particles in a human liposarcoma, J. Nat. Cancer Inst. 44: 407-513, 1970.

3. Morton, D.L., Eilber, F.R., Malmgren, R.A., and Cook, K.O.: Evidence for a virus in human sarcomas. Proceedings 4th International Conference for Comparative Leukemia Research.

4. Morton, D.L., Eilber, F.R., Malmgren, R.A., and Wood, W.C.: Immunologic factors in human malignant melanoma. Surgery 68: 158-164, 1970.

5. Easton, J.M., Rabson, A.S., and Malmgren, R.A.: Transformation of hamster cells in vivo and in vitro by an adenovirus 4-SV40 hybrid. Int. J. of Cancer 5: 15-20, 1970.

6. Barth, R.F., Friedman, R.M., and Malmgren, R.A.: Depression of interferon production in mice following treatment with burro anti-mouse lymphocyte serum. (Abstract). J. Reticuloendothel. Soc. 7: 637, 1970.

7. Sheagren, J.N., Barth, R.F., Edelin, J.B. and Malmgren, R.A.: Mechanism of reticuloendothelial system blockade produced by antilymphocyte serum. J. Immun. 105: 634, 1970.

8. Dolin, R., Blacklow, N.R., Malmgren, R.A. and Chanock, R.M.: Establishment of human fetal intestinal agar cultures for growth of viruses. J. Infect. Dis. 122: 227-231, 1970.

9. Krueger, G.R.F., Malmgren, R.A. and Berard, C.W.: Malignant lymphoma and plasmacytoses in mice with chronic immunosuppression and persistant antigenic stimulation. Transplantation 11: 138-144, 1971.

10. Malmgren, R.A., Morton, D.L. Viral and immunologic studies of human neoplasms. Pathobiology Annual. (In Press)

Serial No. NCI 852
1. Laboratory of Pathology
2. Cytopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Exfoliative cytology applied to human diagnostic problems and research problems.

Previous Serial Number: Same

Principal Investigator: Richard A. Malmgren, M.D.

Other Investigators: Elizabeth W. Chu, M.D. and Costan W. Berard, M.D.*

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total: 9
Professional: 9
Other: None

*From July 1970 to March 1971 Dr. Berard of the Hematopathology Section actively participated in the service functions of the Section

Project Description:

Objectives: The primary responsibility of the Cytopathology Section is to provide the staff of the Clinical Center with an accurate and complete exfoliative cytology service. In addition the Cytopathology Section collaborates in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patient, the course and natural history of the cancer lesion, and the anatomical and physiological changes in the human body associated with various pathologic conditions. The accomplishment of these objectives necessitates the continual improvement of techniques and the origination of new cytologic methods in the laboratory.

The specific objectives include: (1) Development of methods for the automation of cytology. (2) As part of #1, application of the methods being used for automation to study basic normal and malignant cell characteristics. (3) Evaluate methods for automation of cytogenetics and improve chromosome identification. (4) Work, in a general way, to improve the methods of cytopathology including evaluation and development of new or improved methods for cancer detection and diagnosis. (5) Evaluate aspiration biopsy material to develop criteria for cytologic diagnosis of cancer. Material to be obtained from various sites but particularly the breast. (6) Evaluate the cytologic characteristics of normal cellular composition of ileal loop urine where

recurrent cancer may be recognized when it occurs. (7) Conduct experimental studies of possible etiologic factors for carcinoma of the uterine cervix.

Methods Employed: The standard Papanicolaou staining procedure is applied to preparations of various human specimens. These specimens are then screened and evaluated for morphologic changes in the cellular elements. Special procedures involving the use of fluorescent microscopy, microspectrophotometry, millipore filtration, and various special staining and centrifuge techniques are required in many instances. Cultures of patient peripheral blood, bone marrow and tissues are prepared and the chromosome composition analyzed. Coulter counter and cell separation methods are being used.

Patient Material:

The figures compiled below are for the last twelve months.
March 1970 - February 1971

Type of Material Examined	Accessions	Smears
Vaginal and Cervical	1,150	2,042
Other (Ascitic, pleural, gastric, prostatic, urine, bronchial washings, sputa, spinal fluid, sex chromatin study, wound washings, breast, chromosome analysis, chromosome tissue analysis, special procedures	3,750	14,208
Total	4,900	16,250

Institutes	Accessions	Smears
NCI	3,573	12,299
NHLI	291	695
NIAMD	210	601
NIAID	241	544
NINDS	198	726
NIMH	70	160
NIDR	14	61
NEI	47	284
NICH	136	440
Referred	115	432
CC (EHS)	5	8
Total	4,900	16,250

Major Findings: 1. The major contribution of the Cytopathology Section to the diagnostic facilities of the clinical staff has been the early diagnosis of neoplasms of the cervix and lung and of other sites not previously diagnosed. In several instances, exfoliative cytology provided the only tissue confirmation of neoplastic disease before death or treatment of the patient.

2. The second major contribution of the Cytopathology Section is to the research programs of the NIH which require cytologic methods.

3. Methods for cytogenetic studies are now established so that the Cytopathology Section can provide this service on a limited routine basis for Clinical Center studies.

4. The value of exfoliative cytology in examination of aqueous fluid from the eye was demonstrated in a case of metastatic carcinoma of the lung to the eye.

5. Cytologic evaluation of urine from ileal loop patients has been useful in detecting new or recurrent tumors.

Significance: 1. The diagnostic value of exfoliative cytology in the clinical evaluation of cancer and its early detection is readily apparent from the activities of the Cytopathology Section in the Clinical Center and throughout the country. In a number of instances unsuspected *in situ* carcinoma of the cervix has been detected in Clinical Center patients by the routine examination of vaginal and cervical smears on new hospital admission. Exfoliative cytology has often been the only tissue evidence of cancer in a number of patients prior to treatment or death.

2. The need for more cytogenetic studies on a service as well as research basis has existed for some time and now is available.

Proposed Course: The continuation of a complete and adequate cytopathology service for the staff of the Clinical Center is our primary purpose. Continued collaboration with the Clinical Center staff in various research projects and the development of new and improved techniques within the laboratory are an important part of the future program. Particular emphasis will be placed on cytogenetic studies, evaluation of new cancer detection tests and the cytologic characterization of cells aspirated thru a fine needle particularly from breast lesions. A major effort will be placed upon studies to automate cytology.

Work in Progress: In collaboration with Drs. Chu, Rabson, Bamford and Gilfillan human fibroblasts are being exposed to SV40 virus and the % transformed determined. An analysis will be made to determine if any correlation exists between the % transformed and whether or not the patient has a malignancy.

In collaboration with Dr. Chu, mice and hamsters will be treated with oral Enovid and followed by vaginal smear cytology for early changes.

Part of study above, in collaboration with Dr. Hollander of Johns Hopkins, mice will be treated with Trichomonas vaginalis per vagina and half will also receive Enovid.

A study is being made of the blood group antigens in cervical epithelium both normal and neoplastic to see if the antigen loss in neoplastic tissue described by Davidsohn can be confirmed, if it relates to prognosis and if it can be applied to cytologic material.

To study and compare the X-chromosomes in women with and without neoplastic diseases. Collaborative work with New England Medical Center.

In collaboration with Dr. Robert Hoye of the Surgery Branch aspirations thru a fine needle are being evaluated in an effort to establish cytologic criteria which will enable us to make a rapid diagnosis without anesthesia particularly in cases of suspected breast cancer.

Studies are being conducted on methods for improving cell preparation from vaginal and cervical material which will permit the evaluation of these specimens by automated equipment. This is being done in preparation for presentation of these cells to a flow system being constructed by the AEC at Los Alamos.

Publications: 1. Morgan, W.E., Malmgren, R.A., and Albert, D.M.: Metastatic carcinoma of the ciliary body simulating uveitis. Arch. Opthal. 83: 54, 1970.

Serial No. NCI 854
1. Laboratory of Pathology
2. Cytopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Experimental studies on carcinogenesis, endocrine factors and cytogenetic characters

Previous Serical Number: Same

Principal Investigator: Elizabeth W. Chu, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total: 1
Professional: 1
Other: None

Project Description

Objectives:

1. To study the pathogenesis of lesions of rodents after treatment with chemical and biological agents and also to study the influence of these agents on the development of neoplasms and metastasis.

2. To study the possible relationship of X-chromosomes and neoplastic diseases in women.

3. To study effects of Enovid in rodents.

4. To study the effects of Trichomonads in rodents.

Methods Employed:

1. Hamsters were fed with a mixture of ground purina chow containing:

(a) 0.05% DMBA tap water to drink
(b) 0.05% DMBA 5% alcohol to drink
(c) 0.05% DMBA 10% alcohol to drink
(d) 0.05% DMBA 20% alcohol to drink
(e) 0.05% DMBA 30% alcohol to drink

Collaborator: Dr. R. A. Malmgren

2. Peripheral blood lymphocytes and biopsies of skin from patients with and without neoplastic diseases are cultured for chromosomal analysis. Tritiated thymidine is used to label the X-chromosomes for positive identification. The arm lengths of the X-chromosomes of cancer patients are measured and compared with those of non-cancer patients. (In collaboration with Dr. S. Bamford of the New England Medical Center)

3. Three groups of 25 mice in each group are fed with processed Enovid pellets in this study. The Enovid powder is mixed with Gilford's powdered chow and pressed into pellets containing (a) 5 micrograin per gm. of chow, (b) 10 micrograin per gm. of chow and (c) 20 micrograin per gm. of chow.

A fourth group of 20 mice served as contols are given Gilford's chow pellets.

Vaginal smears will be examined at monthly intervals for early detection of neoplastic changes. (Collaborator: Dr. R. A. Malmgren)

4. To study the effects of Trichomonads three groups of mice are set up as follows:

(a) 20 mice will be given 10 micrograms of Enovid per day and 0.1 ml. of Trichomonads in culture medium per vagina at weekly intervals.

(b) 20 mice same as above but given weekly culture medium without the organisms.

(c) 20 mice on daily Enovid only to serve as controls.

Similar groups of Syriam hamsters have been set up but using doses of Enovid double that of mice.

Vaginal smears will be examined at weekly intervals for early detection of neoplastic changes. (Collaborator: Dr. R. A. Malmgren)

Major Findings:

1. The incidence rate of hydrocarbon induced gastrointestinal neoplasm is influenced by the addition of alcohol. The pilot study showed that the incidence rate of the animals using 5% and 10% alcohols as drinking water were 6 to 7 times higher than those using 20% alcohol (66% and 76% as compared to 10%). A final detailed analysis is now being prepared. (Collaborator: Dr. R. A. Malmgren)

2. The pilot study as reported by the New England group of investigators showed a definite difference in the measurements of the sex chromosomes that the X-chromosomes were larger in the tumor bearing females than the non-cancer bearing women.

3. The possible cancer promoting effect on the mice that had been fed with Enovid was reported. The present study is set up to find out the dose effect of Enovid in promoting cancer growth; and also to see if cytological techniques can be applied in the early detection of cancer. The work has been undertaken for about a year. The vaginal smears taken at intermittent intervals on these animals have not shown any remarkable changes. The autopsies performed on a few animals that were found dead showed varying causes of death.

Significance:

1. The promoting effect of alcohol in the induction of cancer by hydrocarbons is noteworthy, especially in the case of DMBA where the neoplastic state is 50% more when 5% or 10% alcohol was used as drinking water as compared to those using 20% alcoholic drinking water. This has practical implications in the case of humans since a large quantity of alcohol is being consumed by many people.

Final analysis of the results is presently underway.

2. Should it be ascertained that the X-chromosomes do measure differently between patients with and without cancers the technique could be applied as a screening method to sort out cancer prone females from the general group of women. Due to factors beyond our control numerous cultures were contaminated. The experiment is being resumed and on new materials.

3. Since millions of American women are put on daily Enovid pills, a definite cancer promoting effect of this drug needs to be further substantiated and investigated more thoroughly.

Proposed Course:

1. To study sex-chromosome differences between tumor-bearing and non-tumor bearing females.

2. Continue to study the chromosomal characteristics of neoplasms that have familial tendencies.

3. Continue to study the effects of Enovid and Enovid in conjunction with Trichomonads.

Publications:

1. Rabson, A.S., Chu, E.W., Berezesky, I.K., Legallais, F.Y., and Grimley, P.M.: Morphologic and cytogenetic studies in vitro of surface-adherent lymphoreticular cells derived from Burkitt's lymphoma tissue. Int. J. Cancer: 5, 217-223 (1970).

2. Yao, K.T.S. and Chu, E.W.: In vitro meiosis of Chinese and Golden hamster oocytes. Radiation Bio-Effects-Summary report. U.S. Dept. of

Health, Education, and Welfare, Public Health Service Publ. No. BRH/DBE 70-1., Washington, D. C., U. S. Government Printing Office, January-December 1969, pp. 202-204.

Serial No. NCI 563
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Mechanisms of Action in Tumor Immunology

Previous Serial Number: Same

Principal Investigator: Costan W. Berard, M.D.

Other Investigators: Gerhard Krueger, M.D.
Richard A. Malmgren, M.D.

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/8
Other: 3/8

Project Description:

Objectives: 1. To study the reactions of murine lymphoreticular tissue to antigenic stimulation.

2. To study the effects of immunosuppressive agents on murine lymphoreticular tissue.

3. To study the effects of combined stimulation and immunosuppression on mouse lymphoreticular tissue.

4. To relate the findings of (1), (2), and (3) to lymphoreticular proliferative disorders of man.

Methods Employed: 1. Antigenic agents of several classes are administered parenterally, either intraperitoneally, intramuscularly, or via footpad injection.

2. Immunosuppressive agents are administered either orally or parenterally.

3. Standard histopathologic techniques, including imprints, smears, and special histochemical reactions, are used to evaluate in vivo reactions.

Major Findings: 1. Principal efforts have been directed at studying the reactions of lymphoreticular tissues of mice in vivo to standardized challenges with vaccination, PPD, LDH virus, and Hela cells. We have shown previously that mice of different strains manifest different patterns and intensity of immune response to various antigens. Balb/c and DBA mice, for example, show a marked proliferation of pyroninophilic transformed lymphocytes in the paracortical areas of the draining lymph nodes 4-7 days after vaccination. In contrast, C3H and C57Bl mice show relatively few such cells but a pronounced hyperplasia of germinal centers.

2. As a sequel to these studies, we have selected Balb/c and C57Bl mice for a long-term study of combined antigenic stimulation (PPD, vaccination, LDH virus, Hela cells) and immunosuppression (Imuran, antilymphocyte serum). We have already determined that:

(a) C57Bl mice are more sensitive to Imuran than Balb/c mice despite the general concept that C57Bl mice are immunologically more "competent" than Balb/c mice.

(b) Chronic administration of antilymphocyte serum induces, in both C57Bl and Balb/c mice, a diffuse lymphoproliferative disorder which is predominantly plasmacytic.

(c) Malignant lymphomas occur in Balb/c mice subjected to chronic antigenic stimulation while on Imuran.

Significance: The lymphoreticular tissues of animals and of man are never static but are instead responding at all times to a host of stimuli of varied types and intensities. Malignant lymphomas are known to be more common in humans with congenital or acquired immunologic disorders and are now being reported with increasing frequency in patients with organ transplants on chronic immunosuppressive therapy. Our studies in mice have been directed at studying the relationship of known methods of antigenic stimulation and immunosuppression to the induction of lymphoproliferative disorders and malignant lymphomas.

Proposed Course: Since the tumor-inducing capability of chronic immunosuppression and continued antigenic stimulation has now been established (see ref. 5), this project is discontinued.

Publications:

1. Graw, Jr., R.G., Rogentine, Jr., G.N., Yankee, R.A., Leventhal, B.G., Whang-Peng, J., Herzig, G.P., Halterman, R.H., Krueger, G., Berard, C.W., and Henderson, E.S.: Spectrum of the graft-versus-host reaction complicating HL-A matched allogeneic bone marrow transplantation. Lancet 2: 1053-1055, 1970.

2. Graw, Jr., R.G., Buckner, C.D., Whang-Peng, J., Leventhal, B.G., Krueger, G.R.F., Berard, C.W., and Henderson, E.S.: Complication of bone marrow transplantation. Graft-versus-host reaction resulting from chronic-myelogenous-leukemia leukocyte transfusions. Lancet 2: 338-341, 1970.

3. Foley, H.T., Herdt, J.R.L., Berard, C.W., and DeVita, V.T.: Analysis of the staging evaluation procedures in 175 patients with Hodgkin's disease. Trans. 10th International Cancer Congress, Medical Arts Publishing Co., Houston, May 1970. Library of Congress #70-124103.

4. Levine, P.H., Ablashi, D.V., Berard, C.W., Carbone, P.P., Waggoner, D.E., and Malan, L.: Elevated antibody titers to Epstein-Barr virus in Hodgkin's disease. Cancer 27: 416-421, 1971.

5. Krueger, G.R.F., Malmgren, R.A., and Berard, C.W.: Malignant lymphomas and plasmacytosis in mice under prolonged immunosuppression and persistent antigenic stimulation. Transplantation 11: 138-144, 1971.

6. Berard, C.W.: Chapter 118, Histopathology of lymphoreticular disorders - conditions with malignant proliferative response - lymphoma. In Principles of Hematology, McGraw-Hill Company, In Press.

7. Carbone, P.P., Ziegler, J.L., Morrow, R., Kyalwazi, S., Brown, C.H., DeVita, V.T., and Berard, C.: Burkitt's Tumor: A comparative study in Africa and the United States. In Recent Results in Cancer Research, In Press.

8. Olweny, C., Ziegler, J., Berard, C.W., and Templeton, A.: Adult Hodgkin's disease in Uganda. Cancer, In Press.

9. Roth, J.A., Siegal, S.E., Levine, A.S., and Berard, C.W.: Fatal recurrent toxoplasmosis in a patient initially infected via a leukocyte transfusion. Amer. J. Clin. Path., In Press.

10. Levine, P.H., O'Conor, G.T., and Berard, C.W.: Antibodies to Epstein-Barr virus (EBV) in American patients with Burkitt's lymphoma. Cancer Res., In Press.

11. Krueger, G.R.F., Berard, C.W., DeLellis, R.A., Graw, Jr., R.G., Yankee, R.A., Leventhal, B.G., Rogentine, Jr., G.N., Herzig, G.P., Halterman, R.H., and Henderson, E.S.: Graft-versus-host disease: Morphologic variation and differential diagnosis in eight cases of HL-A matched bone marrow transplantation. Amer. J. Path., In Press.

12. Krueger, G.R.F., Berard, C.W., Elias, P.M., Graw, Jr., R.G., Rogentine, Jr., G.N., Leventhal, B.G., Yankee, R.A., Herzig, G.P., Halterman, R.H., and Henderson, E.S.: Morphology of graft-versus-host reaction in HL-A matched bone marrow transplantation and its differential diagnosis. Exp. Hemat., In Press.

Serial No. NCI 569
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Project Title: Control of Lymphoma Development in Mice After Dilantin Treatment

Previous Serial Number: Same

Principal Investigator: Gerhard R. F. Krueger, M.D.

Other Investigators: Duran Harris (non-professional)

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/12
Professional: 1/12
Other: 0

Project Description:

The experiment is terminated, a second paper is prepared for publication stating the findings in three early reticulum cell sarcomas as well as a 30% metamorphosis of SJL Hodgkin's-like lesions to reticulum cell sarcoma. No neoplasms were observed in C_3H or C57Bl mice. Immunologic investigations suggest that cellular immune reactions are decreased in dilantin fed mice while humoral reactions appear normal or even increased.

Publications:

1. Krueger, G.: Effect of dilantin in mice. I. Changes in the lymphoreticular tissue after acute exposure. Virchow's Archiv. Abt. A Path. Anat. 349: 297-311, 1970.

Serial No. NCI 572
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Project Title: Immunological Lymphoma Development

Previous Serial Number: Same

Principal Investigator: Gerhard R. F. Krueger, M.D.

Other Investigators: Prof. Dr. M. Nasseri and Dr. H. J. Stolpmann

Cooperating Units: 1. Department of Surgery, Klinikum Westend, Free University (Berlin)
2. Department of Pathology, Electronmikr. Laboratory, Free University (Berlin)

Man Years (July 1, 1970 through June 30, 1971)

Total: 5/6
Professional: 1/3
Other: 1/2

Project Description:

Objectives: This collaborative study involves investigations of the lymphoreticular tissues in transplant recipients (kidney, lung, and bone marrow) of human and animal hosts. Also, investigating different experimental models simulating the effects of transplantation and immunosuppression in mice and dogs as well as monkeys. The ultimate task is to elucidate the cause of lymphoma development in transplant recipients with immunosuppressive therapy.

Methods Employed: Autopsy and biopsy material from human transplant recipients are investigated systematically using histopathological methods. The results are correlated with similar ones from dog and monkey experiments, using Cytoxan, Imuran and antilymphocyte serum as immunosuppressants. In addition, several experiments in mice are continued using light and electron microscopic techniques, transplantation and antigenic stimulation by well defined antigens, immunosuppression by cytostatics, immunosuppressive, Dilantin, thymectomy, and splenectomy. In addition, histologic sections are reviewed from experimental animals under immunosuppressive chemotherapy (obtained from Dr. David Young, Laboratory of Toxicology, National Cancer Institute).

Major Findings: Using above indicated methods, several generalized malignant lymphomas were obtained in different models as well as systemic hyperplasias mimicing neoplasia (see publications). Also, focal reticulum cell aggregates were observed in atrophic lymph nodes and spleens from human allograft recipients as well as from animals with chronic immunosuppression and antigenic stimulation.

Significance: The results have an immediate bearing on human immunopathology and lymphoma development and may influence treatment of immunologic and neoplastic disorders in the future. It is felt that reticulum cell foci in atrophic lymphoreticular tissues as described above may constitute "germ centers" for later lymphoma development.

Proposed Course: To be continued by more extensive use of electron microscopy and determinations of the immunological reactivity of the individual animal.

Publications:

1. Krueger, G.: Attempt of an immunologic interpretation of lymphomagenesis. Dtsch. Med. J. 21: 28, 1970. (original in German).

2. Krueger, G.: Pathogenesis of lymphoreticular neoplasms in transplant recipients. Verh. Dtsch. Ges. Path. 54: 175-181, 1970. (original in German).

3. Krueger, G., Malmgren, R.A., and Berard, C.W.: Malignant lymphomas and plasmacytosis in mice under prolonged immunosuppression and persistent antigenic stimulation. Transplantation 11: 138-144, 1971.

4. Krueger, G: Chronic immunosuppression, persistent antigenic stimulation, and lymphoma development. Presented to the Board of Scientific Counselors, National Cancer Institute, March 22, 1971.

Serial No. NCI 574
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Project Title: Maturation of Lymphoreticular Tissue as Affected by Antigenic Stimulation of the Fetus

Previous Serial Number: Same

Principal Investigator: Gerhard R. F. Krueger, M.D.

Other Investigators: Dr. Hans-Jochen Stolpmann

Cooperating Units: Pathology Institut, Klinikum Steglitz, Free University at Berlin, Germany

Man Years (July 1, 1970 through June 30, 1971)

Total: 3/16
Professional: 1/16
Other: 1/8

Project Description:

This project has been terminated and publications are being prepared.

Publications:

1. Krueger, G., and Stolpmann, H.J.: Postnatal development of lymph nodes in mice after prenatal antigenic stimulation. Presented at VI International Congress of the Reticuloendothelial Society, Freiburg, Germany, July, 1970. Z. Immunitätsforsch (In press).

2. Stolpmann, H.J., and Krueger, G.: The effect of thymectomy on lymphoreticular tissues after prenatal antigenic stimulation. In preparation for publication.

Serial No. NCI 575
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Project Title: Histopathologic Study of Graft-Versus-Host Reaction in Human and Animal Bone Marrow Allograft Recipients.

Previous Serial Number: None

Principal Investigator: Gerhard R. F. Krueger, M.D.

Other Investigators: Robert G. Graw, M.D. and Geoffrey P. Herzig, M.D.

Cooperating Units: Human Tumor Cell Biology Branch and Leukemia Service, Medicine Branch, National Cancer Institute, National Institutes of Health

Man Years (July 1, 1970 through June 30, 1971)

Total: 1/5
Professional: 1/5
Other: 0

Project Description:

Objectives: To investigate the effect of HL-A typing and variable chemotherapeutic measures on the development of graft-versus-host disease. To define graft-versus-host disease morphologically by exacting differential diagnosis. To relate the extent of morphologic changes in graft-versus-host disease to the prognosis of the patient.

Methods Employed: Histopathological methods on materials from human and animal allograft recipients. The animal experiments include extensive controls with transplantation of autologous bone marrow, chemotherapy, administration of antilymphocyte sera, and x-irradiation.

Major Findings:

1. The diagnosis of graft-versus-host is possible histologically, although often difficult to separate from lesions secondary to infection.

2. Graft-versus-host may occur in genotypically matched individuals indicating that loci other than of the HL-A system may be important in the pathogenesis of graft-versus-host.

3. Graft-versus-host reaction may occur despite lack of evidence for graft take indicating that "takes" of the various cell lines transplanted cannot be correlated.

4. Graft-versus-host reaction may occur after transfusion of viable blood cells.

Significance: These results have an immediate influence on future studies to select donor and recipient, and to evaluate the effect of treatment.

Proposed Course: To be continued as indicated; the investigation of human materials is done on a "service basis" involving a large number of investigators from different laboratories.

Publications:

1. Krueger, G., Berard, C.W., DeLellis, R.A., Graw, R.G.,Yankee, R.A., Leventhal, B.G., Rogentine, G.N., Herzig,G.P., Halterman, R.H., and Henderson, E.S.: Graft-versus-host disease. Morphologic variation and differential diagnosis in eight cases of HL-A matched bone marrow transplantation. Amer. J. Path. 63: 179-202, 1971.

2. Krueger, G., Berard, C.W., Elias, P.M., Graw, R.G., Rogentine, G.N., Leventhal, B.G., Yankee, R.A., Herzig, G.P., Halterman, R.W., and Henderson, E.S.: Morphology of graft-versus-host reaction in HL-A matched bone marrow transplantation and its differential diagnosis. Exp. Hematology, (In press). (Presented at Bone Marrow Conference, Exp. Hematology Soc., Pittsburgh, November, 1970).

3. Graw, R.G., Buckner, C.D., Whang-Peng, J., Leventhal, B.G., Krueger, G., Berard, C.W., and Henderson, E.S.: Complication of bone marrow transplantation. Graft-versus-host disease resulting from chronic myelogenous leukemia leukocyte transfusions. Lancet 2: 338-341, 1970.

4. Graw, R.G., Herzig, G.P., Rogentine, G.N., Yankee, R.A., Leventhal, B.G., Whang-Peng, J., Halterman, R.H., Krueger, G., Berard, C.W., and Henderson, E.S.: Graft-versus-host reaction complicating HL-A matched bone marrow transplantation. Lancet 2: 1053-1055, 1970.

Serial No. NCI 576
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Project Title: Histopathologic Study of Side Effects of Cancer Chemotherapy with Special Attention to the Immunosuppressive Effect.

Previous Serial Number: None

Principal Investigator: Gerhard R.F. Krueger, M.D.

Other Investigators: David M. Young, M.D.

Cooperating Units: Laboratory of Toxicology, National Cancer Institute

Man Years (July 1, 1970 through June 30, 1971)

Total: 1/5
Professional: 1/5
Other: 0

Project Description:

Objectives: To investigate the morphogenic effect of several cancer chemotherapeutic drugs on lymphoreticular tissues in order to obtain a simple tool in estimating the immunosuppressive effect of these compounds. Also, to search for unwanted side effects caused by individual drugs.

Methods Employed: Experiments are performed in mice with acute and chronic administration of several chemical immunosuppressants; evaluation is done by histopathological and immunological methods (measurement of cellular and humoral immune response against well defined antigens). The results are correlated with others obtained from morphological investigations of human materials, and of materials obtained from animal experiments done at the Laboratory of Toxicology.

Major Findings:

1. The morphogenic effect of chemical immunosuppressants varies with their dose, the duration of their administration, and with the extent of simultaneous antigenic stimulation.

2. Cell proliferation is not inhibitedin any case of chemical immunosuppression, but in contrast may even be stimulated.

3. No good correlation exists between the depression of antibody formation and the atrophy of lymphoreticular tissues after chemical immunosuppression.

Significance: These findings may have immediate significance with respect to the development of malignant lymphomas in immunologically suppressed individuals (see report No. NCI 572).

Proposed Course: To be continued as indicated above.

Publications:

1. Krueger, G: Morphology of chemical immunosuppression. (In preparation for Advances in Pharmacology and Chemotherapy).

Serial No. NCI 517
1. Laboratory of Pathology
2. Pathological Technology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 - June 30, 1971

Part A:

Project Title: Pathological Technology Section

Principal Investigator: Miss Betty J. Sanders

Other Investigators:

Cooperating Units:

Man Years (July 1, 1970 - June 30, 1971)
Total: 15
Professional: 3
Other: 12

1. Project Description:

Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition developes and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.

July 1, 1970 - June 30, 1971

Number of investigators	85
Number of pieces of tissue	60,401
Number of bottles of tissue	9,968
Number of blocks cut	40,837
Number of blocks cut serially	398
Number of frozen blocks cut	496
Number of blocks recut	983
Number of slides stain H & E	54,768
Number of slides stained special	8,352
Number of unstained slides	3,789
Contract Work - Baker Histology Laboratory	
Number of bottles of tissue	5,678
Number of pieces of tissue	54,225
Number of slides stained H & E	24,219
Contract Work - Hazleton Laboratories, Inc.	
Number of bottles of tissue	1,705
Number of pieces of tissue	24,160
Number of slides stained H & E	9,486

Part A (continued)

2. In addition to the above services, the Pathological Technology Section has had the added workload of cutting and staining 5550 slides for the Dawe-Berard Hematopoietic Neoplasm Workshop to be held in May. These slides will be completed, boxed and mailed by April 1, 1971.

3. The laboratory has been reorganized to provide a central supply for dye solutions to insure quality control and also to save space and money.

4. The administrative duties spent on the contracts have increased more than three fold. In Fiscal Year 1969-70 the man hours used weekly were approximately 16 hours. That time has increased to approximately 50 hours weekly. A poor contractor has added to that burden.

5. Another project now being worked on is the preparation of our techniques for publication. There have been many requests for this at NIH and from researchers elsewhere.

6. We have been asked by Mr. Resnick of the Plant Safety Branch to test the use of Zinc Chloride as a substitute for Mercuric Chloride in fixation of tissues. NIH presently is pouring approximately 50-75 pounds of Mercury in the sewage each year and adding to the pollution. This project is now under evaluation.

Serial No. NCI 853
1. Laboratory of Pathology
2. Surgical Pathology and Postmortem Sections
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Surgical Pathology and Postmortem Sections, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all Institutes in those investigations which involve the use and study of human pathological material. The staff of the Surgical Pathology and Postmortem Sections also carries on independent and collaborative research.

Staff:

Dr. Gregory T. O'Conor, Head, Surgical Pathology Section
Dr. Ralph D. Powell, Jr., Surgical Pathology Section
Dr. Louis B. Thomas, Chief, Laboratory of Pathology and Head, Postmortem Section
+ Dr. Hugh Bonner, Resident
+ Dr. L. Max Buja, Resident
+ Dr. Ronald A. DeLellis, Resident
+ Dr. Elaine Jaffe, Resident
+ Dr. Gerhard Krueger, Resident
+ Dr. Joel A. Roth, Resident
* Dr. Leon Sokoloff, Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases
* Dr. George E. Garrington, Deputy Director of Intramural Research, National Institute of Dental Research
* Dr. William C. Roberts, Chief, Laboratory of Pathology, National Heart and Lung Institute
* Dr. Marius Valsamis, Chief, Diagnostic Neuropathology, Bronx Municipal Hospital, and Assistant Professor, Pathology (Neuropathology), Albert Einstein College of Medicine

---

+ - These physicians are full time Residents in the Laboratory of Pathology.
* - These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Sections.

Project Description:

Objectives: The objectives of the Surgical Pathology and Postmortem Sections are: (1) to provide diagnostic services in pathologic anatomy; (2) to collaborate with National Institutes of Health investigators in research involving use and study of human material; (3) to conduct a residency training program in pathologic anatomy, and (4) to carry out independent research.

1. The service functions of the Sections during the period April 1970 through March 1971 included:

(a) 279 autopsy examinations. Included in this report is a table which shows the number of autopsies performed for each Institute. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held by the staff and residents each morning at 8:30. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the Sections participate.

(b) 5553 specimens were accessioned in the Surgical Pathology Section. A complete listing of these specimens, by Institute, is given in the accompanying table. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research purposes.

(c) Histological preparation and special procedures:

These functions are carried out under the direction of Miss Mary C. Bowling and a complete listing of specimens processed by Institutes is given in the accompanying table.

(d) Photographic Services of the Department

A photographic record is made of a large amount of the pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. Kenzie Edwards who also maintains the equipment and carries out much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, are used extensively for conferences and seminars and many are prepared for publication.

Serial No. NCI 853

REPORT OF SURGICAL PATHOLOGY AND POSTMORTEM SECTIONS APRIL 1, 1970 through MARCH 31, 1971

INSTITUTE AND TYPE OF PATIENT

Service & Type of Activity	NCI	NHLI	NIAMD	NIAID	NINDS	NIMH	NIDR	NEI	NICH	REF	TOTAL
POSTMORTEM PATHOLOGY											
No. of autopsies performed	175	66	13	11	11	0	0	0	1	2	279
Blocks of tissue	9342	2235	843	553	832	0	0	0	70	0	13875
Slides	13178	3159	1727	1007	1149	0	0	0	72	2	20294
Slides actually stained	11575	2713	1226	875	1042	0	0	0	71	40	17542
SURGICAL PATHOLOGY											
Tissue Specimens	4276	270	260	208	106	199	313	23	57	21	5553
Slides H&E stained	17964	913	1474	847	476	59	431	57	243	120	22575
Frozen sections	253	79	64	7	7	0	2	0	4	2	418
Special Procedures	5266	419	449	415	217	16	0	41	59	30	6912

(CONTRACT: 2064 pieces of tissue sent
2095 H&E stained slides returned)

2103

During the past year Mr. Edwards took 2,493 color slides, 424 prints, 1 black and white slide, 1952 black and white negatives, and 144 color rolls of 35 mm. film.

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenburg who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

2. The collaborative activities of the staff and residents in the Surgical Pathology and Postmortem Sections are reflected in the summary of research activities listed below:

(a) Distribution of human organs and tissues

At present there are approximately 35 scientists in the various laboratories at the National Institutes of Health who have requested particular tissues from surgical and postmortem services. On many occasions it is possible to furnish these investigators with fresh human material. A list of these investigators is kept by the secretarial staff and telephone calls are made to the various investigators whenever surgical or postmortem tissues are available.

(b) Conferences:

The staff and residents take an active part in numerous Clinical Center and Institute conferences. In addition, there are several conferences regularly held in the department, including a Surgical Pathology Conference, Neuropathology Conference, Joint Pathologists Conference, Staging Conference, and Gross Autopsy Conference.

A. Dr. Gregory T. O'Conor:

1. Ultrastructure of primary liver cell carcinoma. This is a collaborative study with the Lymphoma Treatment Center in Kampala, Uganda. Liver biopsy specimens have been received and processed. Pilot sections have been cut but electron microscopic study was delayed pending renovation of laboratory.

2. Studies of human and Simian herpes viruses. The oncogenicity and virus cell relationships of these viruses are being investigated in vitro and in vivo. In collaboration with Dr. Alan Rabson, Dr. Ruth Kirschstein, DBS, and Dr. D. Ablashi, VLL, NCI.

3. Electron microscopic study of selected tumor and tumor cell lines. These studies involve screening for viruses and a search for specific morphological changes related to the presence of viral genome.

4. Electron microscopy of selected specimens from the surgical pathology service. The objective is to improve the accuracy of diagnoses in surgical pathology by defining ultrastructural characteristics of specific tissues.

5. Special collaborative studies:

   (a) Electron microscopy of virus induced renal tumor in mice. In collaboration with Dr. George Meyer, Surgery Branch.

   (b) Electron microscopy of plasma cell tumor lines derived from mice. In collaboration with Dr. Donald Buell, Immunology Branch.

   (c) Electron microscopy of penile carcinoma biopsies. In collaboration with Dr. Kiyawalsi, Department of Surgery, Makerere Medical School, Kampala.

6. Collaborative clinicopathologic studies:

   (a) Radiation therapy of lymphosarcoma. In collaboration with the Radiation Branch.

   (b) Geographical variations in Hodgkin's disease, Cali, Colombia and Connecticut. In collaboration with the Biometry Branch.

   (c) Studies in American patients with Burkitt's tumor. With VLL.

7. Review of cancer of the oral cavity and respiratory tract in Uganda. This study is for a proposed textbook under the editorship of Dr. Templeton at the University Medical College in Kampala.

8. Other activities:

   (a) Chairman of Commission on Epidemiology of UICC.

   Responsibility includes coordination of the research program of three committees and the planning of and participation in their meetings. I am actively participating in the investigations in progress by two of these committees, i.e. Childhood Cancer and Hodgkin's disease.

(b) Collaborating center for International Reference Group on Classification of Neoplasms of the Hematopoietic System.

(c) Breast Cancer Steering Committee.

Publications:

1. Senyszyn, J.J., and O'Conor, G.T.: Fibrosarcoma of the brain: A case report describing response of the primary and metastatic tumors to radiotherapy. J. Oncology 24: 431-437, 1970.

2. Primack, A., Wilson, J., O'Conor, G., Engelman, K., Hull, E., and Canellos, G.P.: Hepatocellular carcinoma with the carcinoid syndrome. Cancer (In press).

3. O'Conor, G.T.: Relationship of classification to epidemiology of malignant lymphomas. Proc. Tenth. Int. Cancer Congress, Houston, 1970.

4. Rabson, A.S., O'Conor, G.T., Lorenz, D.E., Kirschstein, R.L., Legallais, F.Y., and Tralka, T.S.: Lymphoid cell culture line derived from lymph node of Marmoset infected with Herpesvirus Saimiri - A preliminary report. J. Nat. Cancer Inst. (In press).

5. Correa, Pelayo, and O'Conor, G.T.: Epidemiologic Patterns of Hodgkin's Disease. Int. J. Cancer. (In Press)

B. Dr. Ralph D. Powell, Jr.:

1. Evaluation of the pathological material available from National Institutes of Health patients as a part of a clinicopathological study of Hodgkin's disease involving the lung.

2. Participation in creation of a WHO Reference Center for Tumors of the Uterus and Placenta.

Publications:

1. Caldwell, E.J., Powell, R.D., Jr., and Mullooly, J.P.: Interstitial emphysema: A study of physiological factors involved in experimental induction of the lesion. Amer. Rev. of Res. Dis. 102: 516-525, 1970.

C. Dr. Louis B. Thomas:

1. Continuing retrospective study of Hodgkin's disease in Clinical Center patients, (With Dr. Costan W. Berard).

2. Continued effort with Dr. Arnold W. Pratt to develop a system for computer coding of pathology diagnoses.

3. Participation in reference center activities for uterine and placental tumors. (W.H.O. Reference Center).

Residents:

D. Dr. L. Max Buja:

1. Biochemical and anatomic studies in patients with medullary carcinoma of the thyroid gland. Collaborator: Dr. Steven Baylin, Experimental Therapeutics Branch, National Heart and Lung Institute.

2. The incidence and significance of coronary arterial thrombi and other observations in fatal acute myocardial infarction. Collaborator: Dr. William C. Roberts, Section of Pathology, National Heart and Lung Institute.

3. Intramitochondrial glycogen deposits in cardiac muscle cells. Collaborator: Dr. V.J. Ferrans, Section of Pathology, National Heart and Lung Institute.

4. Histochemical and electron microscopic studies of basophilic degeneration of myocardium. Collaborator: Dr. V.J. Ferrans, Section of Pathology, National Heart and Lung Institute.

5. Anatomic and functional studies of the acute effects of elective cardiac arrest of canine hearts induced by cross-clamping the ascending aorta. Collaborators: Sidney Levitsky, Department of Surgery, University of Illinois, School of Medicine, Chicago, Illinois, and Dr. V.J. Ferrans, Section of Pathology, National Heart and Lung Institute.

Publications:

1. Berman, M.A., Buja, L.M., and Freed, T.A.: Frothy pink endobronchial secretions following total correction of tetralogy of Fallot. Chest 57: 393-394, 1970.

2. Buja, L.M., Ali, N., Fletcher, R.D., and Roberts, W.C.: Stenosis of the right main pulmonary artery: a complication of acute dissecting aneurysm of the ascending aorta. Amer. Heart J. (In press).

3. Buja, L.M., Levitsky, S., Souther, S.G., Ferrans, V.J., Roberts, W.C., and Morrow, A.G.: Acute and chronic effects of normothermic anoxia on canine hearts: light and electron microscopic evaluation. Circulation.(In press).

4. Levitsky, S., Mullin, E.M., Sloane, R.E., Ferrans, V.J., Buja, L.M. and Morrow, A.G.: Effects of a hyperosmotic perfusate on extended preservation of the heart. Circulation (In press).

5. Ferrans, V.J., Buja, L.M., Levitsky, S. and Roberts, W.C.: Effect of hyperosmotic perfusate on ultrastructure and function of the isolated canine heart. Lab. Invest. (In press).

6. Ferrans, V.J., Buja, L.M., Roberts, W.C., and Frederickson, D.S.: The spleen in type I hyperlipoproteinemia: histochemical, biochemical, microfluorimetric and electron microscopic observations. Amer. J. Path. (In press).

E. Dr. Ronald A. DeLellis:

1. The formaldehyde induced fluorescence technique for the demonstration of biogenic amines in diagnostic histopathology. Will be submitted to CANCER.

2. The detection of metastatic osteogenic sarcoma with strontium 85. In collaboration with Dr. G. Schall, Clinical Center, Nuclear Medicine.

3. Radiologic features of graft versus host disease involving the small intestine. In collaboration with Dr. M. Weinstein, Clinical Center, Radiology.

4. Central nervous system complications in chronic myelogenous leukemia. In collaboration with Dr. M. Shapiro, National Cancer Institute.

5. R.A. DeLellis and J.A. Roth: Histochemical demonstration of norepinephrine in a nonchromaffin functioning glomus jugulare tumor. Submitted to Archives of Pathology.

6. R.A. DeLellis: Iron containing plasma cells in a healed recluse spider bite. Submitted to Archives of Dermatology.

7. G.L. Schall, S.M. Larson, and R.A. DeLellis: Pathogenesis of the positive bone scan and its implications for the detection of metastatic osteosarcomas. Submitted to Journal of Surgical Oncology.

Publications:

1. DeLellis, R.A., and Bowling, M.C.: The use of Sirius red staining in routine histopathology. Human Path. 1: 655, 1970.

2. DeLellis, R.A., Rabson, A.S., and Albert, D.: The cytochemical distribution of catecholamines in the C1300 murine neuroblastoma. J. Histochem. Cytochem. 18: 913-914, 1970.

3. Bowen, R.A., Hassard, D.T.R., Wong, V.G., DeLellis, R.A., and Glenner, G.G.: Lattice dystrophy of the cornea as a variety of amyloidosis. Amer. J. Ophthal. 70: 822-825, 1970.

4. Glenner, G.G., Page D., Isersky, C., Harada, M., Cuatrecasas, P., Eanes, E.D., DeLellis, R.A., Bladen, H.A., and Keiser, H.R.: Murine amyloid fibril protein: isolation, purification, and characterization. J. Histochem. Cytochem. 19: 16-29, 1971.

F. Dr. Gerhard Krueger:

See Individual Reports NCI Nos. 569, 572, 574, 575 and 576.

G. Dr. Joel A. Roth:

1. Light and electron microscopic evaluation of testis biopsies in patients with Hodgkin's disease. In collaboration with Dr. David Van Thiel, National Cancer Institute.

2. Electron microscopic study of brain biopsy in a patient with Schilder's disease. In collaboration with Dr. Simopoulous, National Heart and Lung Institute.

3. F. Enzinger and J.A. Roth: Synovial sarcoma of the pharynx. A clinicopathologic study. Submitted to CANCER.

4. R.A. DeLellis, and J.A. Roth: Histochemical demonstration of norepinephrine in a nonchromaffin functioning glomus jugulare tumor. Submitted to Archives of Pathology.

5. G.L. Schall, J.A. Roth and R. Temple: Differential uptake of $^{125}I^-$ and $^{99}TcO_4^-$ in a histologically unusual metastatic thyroid carcinoma. Submitted to Journal of Nuclear Medicine.

6. A.P. Simopoulous, D. Golde, R.A. Roth, and F.C. Bartter: A new variant of Leigh's encephalopathy: Respiratory alkalosis, hypokalemia, hypomagnesemia and aldosteronism, treated with thiamine, $MgCl_2$ and spironolactone. Submitted to Soc. Rad. Res. Meetings.

7. C. Bagley, J.A. Roth, V. Devita, and L. Thomas: Clinicopathologic correlation of liver biopsies in patients with Hodgkin's disease. Submitted to American Federation for Clinical Research Meetings.

Publications:

1. Bennett, S.H., Futrell, J.W., Roth, J.A., Hoye, R.C., and Ketcham, A.S.: Prognostic significance of implied histologic host response in cancer of the larynx. To be presented at the Ewing Society Meetings, May 6-8, 1971.

2. Roth, J.A., Siegal, S.E., Levine, A.S., and Berard, C.W.: Fatal recurrent toxoplasmosis in a patient initially infected via a leukocyte transfusion. Amer. J. Clin. Path. (In press).

3. Kaufman, H., Ommaya, A., Doppman, J., and Roth, J.A.: Cord syndrome secondary to hypertrophy of the ligamentum flavum in an acromegalic. Arch. Neurol. (In press).

H. Dr. Rolf F. Barth:

Publications:

1. Sheagren, J.N., Barth, R.F., Edelin, J.B., and Malmgren, R.A.: Mechanism of reticuloendothelial system blockade produced by antilymphocyte serum. J. Immun. 105: 634, 1970.

2. Barth, R.F., Willerson, J.T., Buja, L.M., and Roberts, W. C.: Amyloid coronary artery disease, primary systemic amyloidosis and immunoglobulin G. paraproteinemia. Arch. Intern. Med. 126: 627, 1970.

3. Barth, R.F., Grimley, P.M., Berk, P.D., Bloomer, J.R., and Howe, R.B.: Excess lipofuscin accumulation in constitutional hepatic dysfunction (Gilbert's Syndrome). Arch. Path. 91: 41-47, 1971.

4. Barth, R.F., Baker, P.J., Stashak, P.W., and Amsbaugh, D.F.: Enhanced antibody production to type III Pneumococcal polysaccharide in mice treated with antilymphocyte serum: evidence for a cellular regulatory mechanism of the IgM antibody response. Transplantation Proc. (In press).

5. Barth, R.F., Hunter, R.L., and Sheagren, J.N.: Effects of antilymphocyte serum on the localization of 125 I-labelled flagella, colloidal carbon and titanium dioxide in splenic follicles. in Lymphatic Tissue and Germinal Centers in Immune Reactions. Adv. Medicine (In press).

6. Baker, P.J., Stashak, P.W., Amsbaugh, D.F., Prescott, B., and Barth, R.F.: Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to type III pneumococcal polysaccharide. J. Immunol. (In press).

7. Friedman, R.M., Barth, R.F., and Stern, R.: Anomalous effects of heterologous serum on interferon production in mice. Nature (In press).

8. Baker, P.J., Prescott, B., Barth, R.F., Stashak, P.W., and Amsbaugh, D.F.: Immunologic paralysis to type III pneumococcal polysaccharide as assessed by an immunoplaque procedure. Ann. N. Y. Acad. Sci. (In press).

I. Dr. Deborah F. Carpenter:

Publications:

1. Chretien, P.B., Carpenter, D.F., White, N.S., Harrah, J.D., and Lightbody, P.M.: Squamous carcinoma arising in a dentigerous cyst. Oral Surg. 30: 809-816, 1970.

J. Mary C. Bowling:

1. Preparation of color photographs or a color atlas of special stains to supplement laboratory manual.

Publications:

1. DeLellis, R.A., and Bowling, Mary C.: The use of Sirius red and Congo red staining in routine histopathology. Human Path. 1: 655, 1970.

2. Book review on A Guide to Practical Histochemistry. Amer. J. Med. Tech. 37: 27, 1971.

Serial No. NCI 857
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Interstitial Pulmonary Emphysema: Acute and chronic morphological and physiological changes induced by elevation of intratracheal air pressure

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Edgar J. Caldwell, M.D., and John P. Mullooly, Ph.D.

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total: 1/12
Professional: 1/24
Other: 1/24

Project Description:

Objectives:

1. To determine under standard conditions the amount of airway pressure necessary to produce dissection of air into the pulmonary interstitial space.

2. To characterize the lesion morphologically with regard to location, extent, shape, etc.

3. To study the concomitant physiologic changes and determine the pathogenetic significance of such variables as: pulmonary artery pressure, pleural space pressure, lung volume and absolute airway pressure.

4. To expose animals on a chronic basis to elevated airway pressures to determine if such stress will induce emphysema of the type commonly seen in adult humans and to study the evolution of the interstitial emphysema.

5. To compare the lesions occurring in rabbits with those occurring in man to shed light on pathogenetic mechanisms, especially those operating in man.

Major Findings:

1. The incidence of interstitial emphysema is directly related to the applied airway pressure and is high above 50 cm. of water pressure.

2. The incidence of interstitial emphysema increased directly with the degree of expansion (volume) of the lung.

3. The incidence of interstitial emphysema does not seem to be related to the pulmonary artery pressure.

4. The lesion is found less frequently in animals following chronic exposure than following acute exposure at comparable pressures.

5. The lesion in man and rabbits is morphologically very similar.

Significance:

1. In the rabbit, interstitial pulmonary emphysema with significant tissue destruction is induced by airway pressures lower than those normally generated by coughing in the human or than those often created during therapeutic intermittent positive pressure breathing.

2. The observation that the incidence of interstitial emphysema increases with the degree of expansion of the lung, supports the hypothesis that pressure in the interstitial space falls as the degree of expansion of the lung increases, and the hypothesis that the essential cause of interstitial emphysema is the creation of a large pressure gradient between alveoli and the interstitial space.

3. During chronic exposure, the lesion in rabbits appears to heal and the animal becomes more resistant to induction of the lesion.

4. The definite morphological and probably physiological similarities between the lesion in rabbits and in man suggest that preventive measures such as limiting airway pressure elevation and degree of lung expansion may be effective methods of reducing the incidence of the lesion in man.

Proposed Course:

1. Begin in vitro experiments to define more precisely the relationship of lung volume and pulmonary artery pressure to incidence of interstitial emphysema.

2. Develop techniques for measuring pressure in the interstitial space to permit monitoring of this pressure during changes in lung volume, etc.

3. Determine how much and what types of functional disturbances of respiration are produced by interstitial emphysema.

4. Establish more precisely the morphological and physiological similarities between the lesion in man and rabbits.

Publications:

1. Caldwell, E.J., Powell, R.D., Jr., and Mullooly, J.P.: Interstitial emphysema: A study of physiological factors involved in experimental induction of the lesion. Amer. Rev. of Res. Dis. 102: 516-525, 1970.

Serial No. NCI 858
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Induction of ovulation by gonadotropic hormones: A Clinico-pathologic study.

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: John R. Marshall, M.D., Charles B. Hammond, M.D., and Arnold Jacobson, M.D.

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives:

Work on this project has remained suspended during the current year due to Dr. John Marshall's having left NIH but is expected to resume during the coming year.

Serial No. NCI 859
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Renal biopsy in lupus nephritis: A statistical analysis of variability of disease at different sites within single kidneys

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Roger L. Black, M.D., Watson Buchanan, M.D., William M. O'Brien, M.D., and Hugh M. Pettigrew

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives:

Work on this project has been almost completely suspended during the current year but is expected to progress more rapidly during the coming year.

Serial No. NCI 866
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural studies of primary liver cancer

Previous Serial Number: Same

Principal Investigator: Gregory T. O'Conor, M.D.

Cooperating Units: Lymphoma Treatment Center, Kampala, Uganda and Medicine Branch, NCI

Man Years (July 1, 1970 through June 30, 1971)

Total: 1/4
Professional: 1/8
Other: 1/8

Project Description:

Objectives: Correlation of the ultrastructural morphology of primary liver cell tumors with light microscopic findings and with selected clinical and therapeutic parameters.

Methods Employed: Standard electron microscopic techniques. Biopsy material is obtained from patients admitted to the Lymphoma Treatment Center in Uganda and the Clinical Center, Bethesda.

Major Findings: Studies are only now in progress. Work was delayed pending renovation of laboratory.

Significance: Implied in objective.

Proposed Course: Continuation of study.

Serial No. NCI 867
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hodgkin's disease involving the lung

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Martin H. Cohen, M.D., and Vincent P. DeVita, M.D.

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:
Professional:
Other:

Project Description:

Objectives:

Work on this project has been almost completely suspended during the current year, but is expected to progress more rapidly during the coming year.

Serial No. NCI 871
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Light and electron microscopic studies of tumors and cell lines associated with H. saimiri

Previous Serial Number: None

Principal Investigator: Gregory T. O'Conor, M.D.

Other Investigators: Alan S. Rabson, M.D., and Ruth L. Kirschstein, M.D.,

Cooperating Units: Division of Biologics Standards

Man Years (July 1, 1970 through June 30, 1971)

Total: 1/4
Professional: 1/6
Other: 1/12

Project Description:

Objectives:

1. To characterize diseases induced by H. saimiri and related viruses.

2. To investigate the role of H. saimiri in tumor induction.

Major Findings: H. saimiri induces a lymphoreticular proliferative disease in several species of New World monkeys. Two continuous cell lines have been obtained from animals with such tumors. In one of these, immature virus particles can be identified by electron microscopy in an occasional cell; no virus is seen in the other. When these cells are cocultivated with Vero cells mature virus particles are found in abundance.

Significance: This primate model is particularly appropriate for the study of herpes virus oncogenesis and the relationship of an infectious process to neoplasia.

Proposed Course: Continuation of in vivo and in vitro studies with H. saimiri and related herpes viruses in pursuit of the stated objectives.

Serial No. NCI 555
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural and Autoradiographic Studies of Arbovirus Replication

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Robert M. Friedman, M.D., Irene K. Berezesky, B.S., Judith G. Levin, Ph.D., and Nathaniel Young, M.D.

Cooperating Units: Channing Laboratory, Boston City Hospital

Man Years (July 1, 1970 - June 30, 1971)

Total: 1
Professional: 1
Other:

Project Description:

Objectives: To correlate the ultrastructural events in arbovirus development with biochemical studies of viral nucleic acid replication and protein synthesis.

Major Findings: In tissue cultures and experimental animals ultrastructurally unique cytoplasmic vacuoles have been identified in cells infected by group A arboviruses. These vacuoles, designated Type I cytopathic vacuoles (CPV-I), are sites of virus RNA replication. This was demonstrated by high resolution autoradiography and cell fractionation.

As infection progresses arbovirus nucleoids accumulate in the cytoplasm and mature by budding through host cell membranes, either into cytoplasmic vacuoles or endoplasmic cisternae, or at the cell surface. Our studies have demonstrated both pathways of virus envelopment in vivo and in vitro.

Accumulation of virus nucleoids results in the formation of Type II cytopathic vacuoles (CPV-II), probably on the same membranes which are involved in virus protein synthesis (granular endoplasmic reticulum). Studies using metabolic inhibitors demonstrated that a cytoplasmic pool of virus nucleoids persists in cells for up to 2 hours after virus protein synthesis is halted. Since nucleoids form rapidly from nascent protein, this pool

evidently consists of pre-assembled nucleoids which are passively and randomly conveyed to sites of envelopment.

A specific tropism of group A arboviruses for skeletal muscle of young mice was demonstrated. Virus undergoes a single cycle of growth in muscle but does not spread from cell to cell due to trapping of virions at the sarcolemmal basement membrane and within the sarcotubular reticulum. In the central nervous system virus nucleoids are transported long distances in neural processes, and the spread of infection is thus accelerated.

Significance: Identification and study of replicative structures associated with virus development contributes basic information to virology and molecular biology. The group A arboviruses include several important pathogens and studies in progress are providing basic information concerning the pathogenesis of infections.

Proposed Course: The significance and fate of virus replicative vacuoles will be examined in greater detail utilizing cell fractionation techniques, high resolution autoradiography, cytochemistry and conjugated antibodies. The course of virus development will be manipulated with specific inhibitors.

Publications:

1. Grimley, P.M., and Friedman, R.M.: Arboviral infection of voluntary striated muscles. J. Infect. Dis. 122: 45-52, 1970.

Serial No. NCI 555a
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural and Biological Studies of Effects of an Antiviral Drug on Poxvirus Infections

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Bernard Moss, M.D., Edith Rosenblum, B.S., and Sharon J. Mims, B.S.

Cooperating Units: Laboratory of Biology Viruses, NIAID

Man Years (July 1, 1970 - June 30, 1971)

Total: 1 1/2
Professional: 1 1/2
Other:

Project Description:

Objectives: 1. To examine the effects of an antiviral drug on molecular events in poxvirus assembly. 2. To examine the structure and formation of poxvirus membranes. 3. To study poxvirus morphogenesis.

Major Findings: The antibiotic rifampicin blocks poxvirus assembly. Morphogenesis is interrupted at a stage prior to formation of virus envelopes. Viral membrane which accumulate in the presence of rifampicin lack a normal outer coating. When the drug is removed, the morphologic effect is rapidly reversed. Formation of envelopes and immature virus particles precedes an increase in RNA polymerase activity associated with virus maturation. The action of rifampicin is limited to the early stage of morphogenesis. Using metabolic inhibitors of viral nucleic acid or protein synthesis, the events of poxvirus morphogenesis can be dissociated into three major stages: 1) formation of the virus membranes and viroplasmic matrix material; 2) formation of virus envelopes and assembly of immature particles; 3) formation of virus cores and core differentiation.

Significance: The action of the antibiotic rifampicin on virus assembly is unprecedented and it provides a novel approach to the study of virus membrane formation. Poxvirus membranes are unique in that they are assembled de novo in the cytoplasm. Analysis of the poxvirus membrane development can lead to further understanding of membrane bioassembly processes. Studies of

an antiviral agent are also of broad fundamental importance for the development of other therapeutic agents.

Proposed Course: The effects of rifampicin and metabolic inhibitors on various stages of virus development will be examined. The action of the drug will be probed using a mutant virus. Attempts will be made to localize virus nucleic acids, proteins and lipids by ultrastructural techniques. Efficacy of rifampicin analogs will be tested.

Publications:

1. Katz, E., Grimley, P., and Moss, B.: Reversal of the antiviral effects produced by rifampicin. Nature 227: 1050-1051, 1970.

2. Grimley, P.M., Rosenblum, E.N., Mims, S.J., and Moss, B.: Interruption by rifampicin of an early stage in vaccinia virus morphogenesis. Accumulation of membranes which are precursors of virus envelopes. J. Virol. 6: 519-533, 1970.

Serial No. NCI 577
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural and Biologic Studies of Endoreticular Tubules in Neoplastic and Virus-infected Cells

Previous Serial Number: None

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Theodore Pincus, M.D.

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/2
Other:

Project Description:

Objectives: 1. To compare the ultrastructure of endoreticular tubules in neoplastic and virus-infected cells.

2. To correlate appearance of the tubules with cell functions.

Major Findings: Endoreticular tubules of similar morphology were observed in lymphoma cells, glomerular endothelium and cells infected by arboviruses. The tubules consistently measured 220-240 Angstroms in diameter, but varied in electron density. They were always within the granular endoplasmic reticulum or perinuclear cisternum.

Significance: The endoreticular tubules are often observed in cells of patients with systemic lupus erythematosus or Sjögren's disease as well as in lymphoid cell cultures derived from normal, leukemic, cystinotic and lymphoma patients. Thus, they appear to have a broad biologic significance, and may be related to virus infections or transformation.

Proposed Course: Attempts will be made to induce formation of the particles, isolate them and characterize them by autoradiographic or labelled antibody techniques.

Publications:

1. Pincus, T., Blacklow, N.R., Grimley, P.M., and Bellanti, J.A.: Glomerular microtubules of systemic lupus erythematosus. Lancet 2: 1058-1061, 1970.

Serial No. NCI 861
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural, Histochemical and Biological Studies of Hormonally Active Tumor Cells

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: William Hammond, M.D., and Nelson A. Wivel, M.D.

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/4
Professional: 1/4
Other:

Project Description:

Objectives: 1. To study the histology and ultrastructure of paragangliomas, pheochromocytomas, and related endocrine tumors (multiple endocrine neoplasia, Type II).

2. To study clinical, histochemical and biochemical differences in normal and neoplastic endocrine cells.

3. To develop appropriate experimental systems for the study of endocrine neoplasms.

Major Findings: Combined ultrastructural, histochemical and fluorometric evidence suggests that tumors derived from the carotid body and other paraganglia produce catecholamines. Common ancestry of paraganglia and chromaffin ganglia was suggested by electron microscopic comparisons. Findings in the tumors provide a better basis for predicting the mechanism of chemosensation in normal paraganglia.

Medullary carcinoma of the thyroid gland is associated with an unusual incidence of pheochromocytomas. Cells of this tumor were established in culture and production of thyrocalcitonin and prostaglandins was demonstrated in vitro. These cells were transformed by simian virus 40 which enhanced their growth in vitro. The cells continued to display specific activities.

Significance: Tumors of the neuroendocrine ganglia are of unusual biological interest, since the synthesis of a specific product and presence of unique cytoplasmic enzymes provide a persistent marker after neoplastic transformation. Study of multiple endocrine edematosis Type II and the relationship of pheochromocytoma to thyroid carcinoma may elucidate humoral influences in endocrine neoplasia.

Proposed Course: To continue tissue culture of medullary thyroid carcinoma cells, determine the persistence of hormone production in vitro and examine specific enzyme activities.

Serial No. NCI 863a
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural Studies of Human Surgical and Cytology Specimens

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Richard A. Malmgren, M.D., Irene K. Berezesky, B.S., and Herman J. Michelitch, B.S.

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/2
Professional: 1/2
Other:

Project Description:

Objectives: 1. To compare the ultrastructure of normal and neoplastic cells in surgical specimens or samples of body fluids.

2. To compare the ultrastructural features of tumor cells grown in vitro with samples of the original materials.

3. To identify the presence, if any, of particles resembling viral etiologic agents.

Major Findings: Methods for routine preservation of surgical tumor specimens for ultrastructural examination have been improved and methods for the concentration and electron microscopic examination of cells in body fluids have been developed. Preliminary correlation of electron micrographs with cytology specimens suggests that histiocytes and mesothelial cells in pleural fluids may be distinguished by ultrastructural features.

Significance: The significance of such investigations lies in the stated objectives: Results contribute basic information regarding tumor ultrastructure and etiology and may assist in cytodiagnosis.

Proposed Course: As stated in objectives.

Serial No. NCI 863b
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural and Biologic Studies of Cells Infected with Herpes-group Viruses

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Alan S. Rabson, M.D., and Irene K. Berezesky, B.S.

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1/4
Professional: 1/4
Other:

Project Description:

Objectives: 1. To examine ultrastructural features of cell lines displaying "herpes-like" intranuclear particles; specifically, lymphoid cell lines derived from patients with malignant lymphoma and other diseases.

2. To study the growth cycle and biologic effects of herpes-group viruses in lymphoma and other cell lines.

Major Findings: 1. The morphologic effects of herpes simplex virus infection in lymphoma cells are quite similar to changes apparently induced by the spontaneously occurring "herpes-like" or EB virus particles. These changes include disfigurement of the nuclear envelope and a general alteration of cytomembrane organization.

2. Cells infected by a rat cytomegalovirus contain unusual nuclear fibers and cytoplasmic masses of virus cores. The rat virus grows in rat and hamster cells. In rat cells, virus may develop in cytoplasmic "factories".

3. Cultured lymphoid cells, transformed by EB virus appear to interact *in vitro* and perform the function of emperipolesis.

Significance: Comparison of cytologic reactions to infection by herpes-type viruses may elucidate the significance of EB agents detected in human lymphoid cell lines cultured *in vitro*. In addition the studies outlined contribute basic biologic information in the area of virus-host cell relationships.

Proposed Course: 1. To continue ultrastructural and biologic comparisons of herpes-group viruses and their effects on host cell membranes.

2. To study experimental infections with herpes-group viruses.

Serial No. NCI 524
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Interferon Production and Action

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman, M.D.

Other Investigators: Maureen Myers

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: To study the mechanism of action of interferon and its possible role in viral oncogenesis.

Methods Employed: Standard virologic methods for virus and interferon assay. Standard biochemical assays for protein and RNA which involve the use of radioisotopes, radioautography, base ratio analysis, sucrose density gradient analysis, electron microscopy, and polyacrylamide gel electrophoresis.

Major Findings: 1. We have been studying control over interferon production in human cells in culture. In brief, superinduction of interferon occurs in this system in the presence of low doses of actinomycin D (0.1 to 0.3 μg/ml) or of high doses of cycloheximide. On the other hand puromycin and fluorophenylalanine completely inhibit interferon production. In a cell-free mitochondrial preparation incubated at 37°, interferon appears. The origin of this interferon is now being studied.

2. Several sorts of heterologous serum inhibit interferon production in mice stimulated by poly IC. This appears to be due to the presence of an unusual nuclease in these sera. The activity of this enzyme is to hydrolyze double-stranded RNA.

3. We have been studying some of the effects of metabolic inhibitors of interferon action. We have discovered an agent (MPB) which appears to block membrane associated biological reactions and to inhibit interferon action.

Significance: 1. A previously undetected step in interferon action may be revealed by studies with MPB.

1A. The control over interferon production in human cells appears to be quite complex. One phase of it seems to be a protein whose action is to inhibit interferon production in cells which have not been induced. Also, the site of interferon production has many of the metabolic characteristics of the mitochondria.

1B. A method for the preparation of high titers of human interferon from diploid human cells has now been developed with the introduction of antimetabolites.

2. The enzyme which hydrolyzes double-stranded RNA appears to be of some importance in shaping the interferon response of animals to inoculation of poly IC.

Proposed Course: 1. We are attempting to find what sort of cell structure human interferon is produced on.

2. We hope to set up a cell-free protein synthesizing system to understand interferon action.

Publications:

1. Friedman, R.M., and Sonnabend, J.A.: Mechanism of action of interferon. Arch. Int. Med. 26: 51-63, 1970.

2. Friedman, R.M.: Interferon inhibition of protein synthesis directed by the genome of parental Semliki Forest Virus. In Nagano, Y., and Levy, H.B. (Eds.): Interferon. Tokyo, Japan, Igaku Shoin Ltd., 1970, pp. 44-57.

3. Friedman, R.F., and Sonnabend, J.A.: Factors which modify interferon assay in chick cells. Internat. Symp. on Standardization of Interferon and Interferon Inducers, London, 1969. Symp. Series Immunobiol. Standard. 14: 125-130, 1970.

4. Friedman, R.M.: Studies on the mechanism of interferon action. J. Gen. Physiol. 56: 149-171, 1970. (Special Supplement)

5. Friedman, R.M.: Studies on the mechanism of interferon action. Proc. of the New York Heart Assoc. Symp. on Interferon, 1969. In Vilcek, J. (Ed.): Interferon. New York, New York, Little, Brown, 1970, pp. 149-171.

6. Friedman, R.M.: Interferon inhibition of protein synthesis directed by the genome of input Semliki Forest virus. In L'Interferon. Publication No. 6; Colloques de L'Institut National de La Santé et de La Recherche Médicale; Minister of Public Health and Social Security, 1970, pp. 101-118.

7. Friedman, R.M., Guggenheim, M.A., and Rabson, A.S.: Interferon action in heterokaryons. In L'Interferon. Publication No. 6; Colloques de L'Institut National de La Santé et de La Recherche Médicale; Minister of Public Health and Social Security, 1970, pp. 153-158.

8. Friedman, R.M., and Pastan, I.: Potentiation of interferon's antiviral activity by cyclic-3'5'-adenosine monophosphate. In L'Interferon. Publication No. 6; Colloques de L'Institut National de La Santé et de La Recherche Médicale; Minister of Public Health and Social Security, 1970, pp. 213-218.

9. Friedman, R.M., Barth, R.F., and Stern, R.: Anomalous effects of heterologous serum on interferon production in mice. Nature, In Press.

10. Sonnabend, J.A., and Friedman, R.M.: Mechanism of antiviral action of interferons. In Finter, N.B. (Ed.): The Interferons. (Ed. 2), In Press.

11. Friedman, R.M., and Buckler, C.E.: Interferons. In Bauer, J. (Ed.): International Encyclopedia of Pharmacology, In Press.

12. Friedman, R.M., and Nebert, D.W.: Viral interference: The mechanism of action of interferon. Symposium on Drugs and Cell Regulation, Academic Press, In Press.

Serial No. NCI 531
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In Vitro Studies of Human Malignant Lymphomas, Sarcomas and Retinoblastoma

Previous Serial Number: Same

Principal Investigator: Alan S. Rabson, M.D.

Other Investigators: P.M. Grimley, M.D., G.T. O'Conor, M.D., E.W. Chu, M.D., and Daniel Albert, M.D. (Yale University)

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: To study biological properties of human lymphoma, sarcoma, and retinoblastoma cells in vitro and to attempt to isolate oncogenic viruses from these cells.

Methods Employed: Standard tissue culture, virologic and pathologic techniques are used.

Major Findings: a. Cell lines have been established from a number of sarcomas and lymphomas of Clinical Center patients and these are being studied with a variety of techniques in attempts to demonstrate and isolate oncogenic viruses. No viruses have been isolated thus far.

b. Studies of ocular tumors including retinoblastomas and malignant melanomas in collaboration with Dr. Albert (Yale University) have continued. Attempts have been made to demonstrate viruses in these tumors by electron microscopy and a variety of virologic techniques but these have not been successful as yet. Catecholamines have been searched for in human retinoblastomas in collaboration with Dr. DeLellis and none were found but they were readily demonstrable in cultures of a murine neuroblastoma.

Significance: The importance of the search for possible viral etiology of human malignant lymphomas is evident. In addition, any information about the biological properties of human lymphoma cells may be of importance in our understanding of the lymphoma problem.

Proposed Course: Attempts to isolate viruses from human malignant tumors using a variety of techniques which have been successful in the isolation of oncogenic viruses in animal systems will be continued.

Publications:

1. Albert, D.M., Dalton, A.J., and Rabson, A.S.: Microtubules in retinoblastomas. Amer. J. Ophthal. 69: 296-299, 1970.

2. Rabson, A.S., Chu, E.W., Berezesky, I.K., Legallais, F.Y., and Grimley, P.M.: Morphologic and cytogenetic studies in vitro of surface-adherent lymphoreticular cells derived from Burkitt's lymphoma tissue. Int. J. Cancer 5: 217-223, 1970.

3. Friedman, R.M., Guggenheim, M.A., and Rabson, A.S.: Interferon action in heterokaryons. In L'Interferon. Publication No. 6; Colloques de L'Institut National de La Santé et de La Recherche Médicale; Minister of Public Health and Social Security, 1970, pp. 153-158.

4. DeLellis, R.A., Rabson, A.S., and Albert, D.: The cytochemical distribution of catecholamines in the C1300 murine neuroblastoma. J. Histochem. Cytochem. 18: 913-914, 1970.

Serial No. NCI 532
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Oncogenic DNA Viruses

Previous Serial Number: Same

Principal Investigator: Alan S. Rabson, M.D.

Other Investigators: R.A. Malmgren, M.D., R.L. Kirschstein, M.D., and G.T. O'Conor, M.D.

Cooperating Units: None

Man Years (July 1, 1970 - June 30, 1971)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: To study some biological properties of DNA viruses with proven or possible oncogenic activity.

Methods Employed: Standard virologic and pathologic methods have been used.

Major Findings: 1. Intensive studies of an oncogenic herpesvirus of New World primates, Herpesvirus saimiri, have been initiated. Marmosets have been inoculated with the agent and lesions resembling malignant lymphomas have been produced, confirming the work of Melendez and his associates who first isolated and described the agent.

A lymphoid cell line has been isolated from a lymph node of a marmoset infected with Herpesvirus saimiri with lymphoreticular proliferative lesions. By electron microscopy a small number of the cells in culture contain immature herpesvirus particles. When the marmoset lymphoid cells are co-cultivated with monolayers of green monkey kidney cells a herpesvirus can be isolated from 25 to 50% of the cells. This herpesvirus appears to be latent or repressed in the lymphoid cells and can be released on contact with appropriate indicator cells. The herpesvirus released, however, differs in a number of ways from Herpesvirus saimiri and may represent a related marmoset herpesvirus or a variant of H. saimiri.

2. In attempts to clarify the virus-cell relationship in cell lines derived from Burkitt's lymphomas which contain small amounts of EB virus, we have attempted to demonstrate virus inhibitors in these cells.

An inhibitor of Herpesvirus hominis has been found in extracts of Burkitt's lymphoma cells. This material is active in plaque inhibition and growth inhibition experiments in vitro and does not appear to be interferon, an interferon inducer or viral antibody. In collaboration with Dr. D. Albert of the Department of Ophthalmology of Yale University School of Medicine this material has been tested in vivo in herpes keratitis in rabbits and has been found to inhibit the progression of lesions.

Significance: The mechanisms of repression and possible integration of herpesviruses in mammalian cells and mechanisms of neoplastic transformation may be of extreme importance in understanding the role of herpesviruses in human cancer. Studies with a model system involving a primate oncogenic herpesvirus such as H. saimiri and studies of herpesvirus inhibitors in human lymphoma cells may be of value in this regard.

Proposed Course: We intend to continue studies of the virus-cell relationship in the marmoset lymphoid cell line and to study factors involved in activation of the viral genome. We intend to continue studies of the herpesvirus inhibitor in human lymphoma cells with hopes of further characterizing it.

Publications:

1. Rabson, A.S., Legallais, F.Y., and Tyrrell, S.A.: An inhibitor of Herpesvirus hominis in extracts of cultures of Burkitt's lymphoma cells. Proc. Soc. Exp. Biol. Med., In Press.

2. Berezesky, I.K., Grimley, P.M., Tyrrell, S.A., and Rabson, A.S.: Ultrastructure of a rat cytomegalovirus. Exp. Molec. Path., In Press.

3. Rabson, A.S., O'Conor, G.T., Lorenz, D.E., Kirschstein, R.L., Legallais, F.Y., and Tralka, T.S.: Lymphoid cell culture line derived from lymph node of marmoset infected with Herpesvirus saimiri - A preliminary report. J. Nat. Cancer Inst., In Press.

Serial No. NCI 554
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Replication of an RNA Virus

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman, M.D.

Other Investigators: Philip M. Grimley, Robert Stern, Ira Pastan, J. Levin, T.K. Yun, and T. Sreevalsan

Cooperating Units: NIDR; Georgetown University Medical School

Man Years (July 1, 1970 - June 30, 1971)

Total: 1
Professional: 1 1/2
Other: 1/2

Project Description:

Objectives: To study the replicative process of an RNA virus.

Methods Employed: Viral plaque assays. RNA extractions. Sucrose gradients. Scintillation counting. Electron microscopy. Base ratio analysis. Lipid analysis. Column chromatography. Acrylamide gel electrophoresis.

Major Findings: 1. We have analyzed the RNA forms of an arbovirus (Semliki Forest virus - SFV) and found that at least 9 forms exist in infected cells. Of these, 4 are single-stranded, 4 are double-stranded and others represent intermediates between these.

2. Several membraneous structures have been found in SFV infected cells. The morphogenesis and function of these is being investigated. One of the structures seems to be a membrane associated replication complex.

3. During the course of these studies we have found a double-stranded RNA form in normal cells. This structure may be important in the transcription process.

Significance: 1. These studies provide the beginnings of an understanding of cellular RNA synthesis.

2. These studies may provide some basis for an understanding of membrane function.

3. These studies may have important implications in understanding the transcription process in animal cells.

Proposed Course: 1. We are attempting to understand the processes of RNA and protein synthesis in SF virus infected cells and their association to membranes.

2. We are also attempting to define what the relationship between the double-stranded RNA we find in animals and the rapidly labeled heterologous nuclear RNA might be.

Publications:

1. Stern, R., and Friedman, R.M.: Apparent double-stranded RNA synthesized in animal cells in the presence of actinomycin D. Nature 226: 612-616, 1970.

2. Grimley, P.M., and Friedman, R.M.: Arboviral infection of voluntary striated muscles. J. Infect. Dis. 122: 45-52, 1970.

3. Friedman, R.M., and Sreevalsan, T.: Membrane binding of input arbovirus ribonucleic acid: Effect of interferon or cycloheximide. J. Virol. 6: 169-175, 1970.

4. Levin, J.G., and Friedman, R.M.: Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol., In Press.

Serial No. NCI 567
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on a Sarcoma Virus

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman, M.D.

Other Investigators: Andrew Horvath and George Johnson

Cooperating Units: NIDR

Man Years (July 1, 1970 - June 30, 1971)

Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: To study the structure and function of the murine sarcoma virus (MSV).

Methods Employed: Virus purification, RNA and protein analysis. Analysis of viral RNA.

Major Findings: 1. Replication processes of RNA tumor viruses. We have been interested in the forms of viral RNA produced by murine sarcoma and leukemia viruses. With Dr. Horvath we have purified the murine sarcoma virus and investigated some of its properties. We have also extracted and analyzed its RNA and its proteins.

2. We have studied the fusion process in XC cells following infection with members of the RNA tumor virus group.

3. During the course of these studies we have found a marked morphologic alteration in animal cells during their incubation with cyclic AMP.

Significance: 1. The findings provide a basis for further work on RNA tumor viruses.

2. The findings may provide a basis for a rapid assay for RNA tumor viruses. Also, they may help to understand some of the events which occur early in the infection process with these agents.

3. These findings may be important in understanding the control of cell morphogenesis and growth.

Proposed Course: We have had to end work in this area because not enough personnel are available in my laboratory to follow up the above findings.

Publications:

1. Johnson, G.S., Friedman, R.M., and Pastan, I.: Analysis of the fusion of XC cells induced by homogenates of murine leukemia virus-infected cells and by purified murine leukemia virus. J. Virol., In Press.

2. Horvath, A.E., and Friedman, R.M.: Nucleic acid and proteins isolated from a strain of murine sarcoma virus (MSV-0). Proc. Soc. Exp. Biol. Med., In Press.

3. Johnson, G.S., Friedman, R.M., and Pastan, I.: Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3'-5'-cyclic monophosphate and its derivatives. Proc. Nat. Acad. Sci. 68: 425-429, 1971.

Serial No. NCI 578
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Molecular Mechanisms Controlling the Differentiation of Normal and Malignant Cells

Previous Serial Number: NCI 3244

Principal Investigator: Phillip Periman, M.D.

Other Investigators: Dr. Behzad Mohit, Dr. Alan Rabson, Dr. James Rose, and Dr. Fred Mushinski

Cooperating Units:

Man Years (July 1, 1970 - June 30, 1971)

Total:
Professional: 1/4
Other:

Project Description:

A. The selective expression of the immunoglobulin genes: a hybrid cell study.

Neoplastic plasma cells adapted to grow in cell culture secrete immunoglobulins with antibody activity (see ref. 1). Each cell line produces a unique immunoglobulin. Some defective cell lines make only light chains. Since the advent of viral induced cell fusion with the subsequent production of proliferating hybrid cell lines, it has seemed likely that some elucidation might be obtained of the mechanisms controlling the synthesis of immunoglobulins. To this end antibody producing cell lines have been fused to a cell line not synthesizing immunoglobulins (see ref. 2). The hybrid cell lines made antibody, but in a much reduced amount.

In order to determine if the reduction in immunoglobulin production was a peculiarity of the non-producing line, hybrid cells have been made between plasma cell lines and several other non-producing cell lines, e.g. an embryonic cell line (3T3) and an epithelial cell line (a renal adenocarcinoma). Analysis of these hybrid cell lines is in progress.

A plasma cell line makes only one of several possible immunoglobulin heavy chains. In collaboration with B. Mohit hybrid cells are to be produced between a gamma A producing cell line and a gamma G producing cell line. The

selection of the hybrid cell line depends upon the isolation of mutant cell lines which are resistant to certain antimetabolites, e.g. Budr and 8 azaguanine; yet, remain sensitive to aminopterin. Such cell lines have been isolated in our laboratories. The production of hybrid cell lines between these mutant plasma cells is in progress.

B. A search for the "oncogene".

Recent studies of malignancy in hybrid cells (Harris, H. et. al., J. Cell Sci., in press) suggest that certain chromosomes carry the information which causes a cell to be malignant. I have fused a malignant hamster cell line whose chromosomal complement is almost exclusively metacentric with a non-malignant primary mouse embryo cell line. These cells have been injected into mice. All cells with a large element of hamster chromosomes will be rejected because of the immunological differences. It is hoped that a few cells will carry only the hamster information for malignancy. These should grow. Chromosomal analysis may reveal the particular hamster chromosome carrying the information. If successful, further experiments using hyperplastic and neoplastic breast tissue will be examined in a similar fashion. It may be possible to detect a chromosomal trait for malignancy.

C. The intracellular localization of an infecting virus.

Recently, it has become possible to identify specific regions of DNA using the technique of in situ DNA-RNA hybridization (Pardue, M.L., and Gall, J.G., Science 168: 1356, 1970). In collaboration with Jim Rose and Al Rabson, I am examining by in situ hybridization cells infected with DNA viruses. These experiments are in progress. If successful, we plan to search for viral genomes in transformed cell lines.

D. The chromosomal association of a carcinogenic DNA virus in hybrid cells.

The exact relationship between a transforming virus and the host cell genome is not known. One study suggests that all host chromosomes have an associated viral genome (Weiss, M.C., Proc. Nat. Acad. Sci. 67: 1164, 1970). Since Al Rabson has developed a plaque assay which detects the presence of a DNA virus (H. saimiri) in a continuous line of Marmoset lymphoid cells, it may be possible to verify and extend Weiss's observations with SV 40. We have made hybrid cells between a mouse epithelial cell line and his Marmoset cell line. These experiments are in progress. Several hybrid colonies will be examined to see if any relationship between chromosomal content and viral production exists.

E. tRNA variations and protein synthesis.

Mushinski has demonstrated that a plasma cell tumor has its own chromatographic set of tRNA molecules (Biochemistry 8: 1684, 1969). The relationship between this variation and the amino acid sequences of immunoglobulins made by these tumors is unknown. In collaboration with Fred Mushinski, the

antibody made by a plasma cell line (MOPC 315) is being compared with that made by the tumor. Preliminary data suggests that the variations in tRNA patterns between tumor and cell line are not responsible for any variation between the antibody made by that tumor and that made by the cell line derived from that tumor.

Publications:

1. Periman, P.: Cell culture of three antibody-producing murine plasmacytomas. J. Nat. Cancer Inst. 46: 403-410, 1971.

2. Periman, P.: IgG synthesis in hybrid cells from an antibody producing mouse myeloma and an L cell substrain. Nature 228: 1086-1087, 1970.

Computational services. Applications programming for, and use of, the IBM-1620 computer has continued for a variety of research problems. There has been particular emphasis on programming and computation to handle data from double label experiments with scintillation counter output, as in, for example, studies of biosynthetic mechanisms. The computer is used by the individual scientific investigator to reduce, calculate and plot such data. Other applications of the computer include its use in areas of research requiring statistical analysis and curve fitting, for example, in the analysis of enzyme kinetics. In some instances, use has been made of the central NIH computer facility.

Ultrastructural localization of thyroperoxidase in the thyroid follicle. Iodination of thyroglobulin appears to occur in the lumen of the thyroid follicle, the site of almost all the thyroid iodoprotein. A thyroperoxidase is known to be able to iodinate thyroglobulin and to effect the synthesis of thyroxine from diiodotyrosine in peptide linkage. Thyroperoxidase has now been observed on the apical plasma membrane where it can come in contact with its stored substrate and make possible the extracellular synthesis of thyroid hormone.

Kinetics of iodine turnover in individual thyroid follicles. A theoretical analysis has been made of experimental data obtained on the uptake and release of iodine by individual follicles. There appear to be two pools of iodine in each follicle, one of which turns over rapidly, the other slowly. Uptake and release of rapidly turning over iodine is proportional to the surface area of the follicular lumen. The quantitative properties of the follicle are determined by the number of cells per follicle. The slow pool of iodine amounts to approximately 30% of the thyroid iodine and turns over at a rate proportional to the volume of the follicular lumen.

Control of globin synthesis by hemin. The maintenance of the optimal rate of hemoglobin synthesis in both intact rabbit reticulocytes and in cell-free preparations is determined by the availability of hemin. In both systems, new globin chains fail to be initiated when hemin is not available. In the cell-free system an inhibitor of globin chain initiation is produced in the absence of hemin. This inhibitor is composed of one or more proteins and potent preparations can be made from preexisting protein by incubation of the ribosome-free supernatant fraction of reticulocytes. Although hemin blocks the formation of the inhibitor, it does not reduce the potency of preformed inhibitor. Inhibitor potency can be reduced by an extract of reticulocyte ribosomes, suggesting that the inhibitor may compete with a natural factor. The inhibitor does not appear to be globin or a by-product of globin. In the cell-free system the crude inhibitor blocks the α- and β-chains of hemoglobin equally, and also inhibits the synthesis of minor protein component(s) which appear to be unrelated to globin or hemoglobin.

Urinary excretion patterns in leukemia. A program is being written to find the best set of wavelengths for the analysis of a multicomponent system from its absorbance spectrum. A program has also been written for the 1620 plotter to allow one to produce complex computer-drawn diagrams.

Replication of DNA. As part of a continuing study of the process of replication of DNA in dividing HeLa cells, the major effort has involved further characterization by various physicochemical techniques of the newly-formed deoxynucleoprotein particles, and of the ways in which they differ from analogous preexisting material. From changes that occur following partial denaturation of the DNA or protein components, or after selective removal of certain of the proteins, it appears that many of the unique differences between newly-formed and preexisting nucleoproteins are due to conformational changes that involve a class of easily dissociable protein, and that the differences can be obliterated by removal of this class of protein.

Studies on methionineless death. This study has been concerned with the observation that methionine auxotrophs of Escherichia coli strain 15 lose their colony-forming ability when deprived of this amino acid. The basis for this phenomenon has now been found to be due to a restrictive locus, $\Gamma 15^+$. The restriction gene product, presumably an endonuclease, cleaves at unmethylated regions of the chromosomal DNA that are synthesized during methionine starvation.

Pseudouridine metabolism in E. coli. A new enzymatic pathway has been uncovered in E. coli for the utilization of pseudouridine as a pyrimidine source. The pathway consists of the following coupled reaction:

$$1)\ \text{pseudouridine} + \text{ATP} \xrightarrow[\text{kinase}]{\text{pseudouridine}} \text{pseudouridylate}$$

$$2)\ \text{pseudouridylate} \xrightarrow[\text{synthetase}]{\text{pseudouridylate}} \text{uracil} + \text{ribose-5-P}$$

Both enzymatic activities are catabolite repressible. Cyclic AMP prevents catabolite repression.

Tyrosine aminotransferase. The mechanism by which the activity of the enzyme, tyrosine aminotransferase, is regulated in embryonic liver is being investigated. It has been found that in vitro acid and alkaline phosphatases are capable of dephosphorylating the cofactor of the enzyme, pyridoxal phosphate (a vitamin B_6 derivative). The apoenzyme is extremely labile and rapidly becomes inactivated. The role of phosphatase in degrading tyrosine aminotransferase in vivo is being studied by means of embryonic liver cells in tissue culture.

Collagen synthesis. The regulation of collagen synthesis in various tissues of the developing chick embryo is being studied. The development of bone tissue appears to involve the transformation of cells from those that produce cartilage to those that produce ossified bone matrix. Concommitant with the change in cell type, there is an increase in the amount of collagen synthesized by bone cells relative to other proteins. The factors which influence this transformation are being investigated.

Energy metabolism. The work of the laboratory has been on whole animal regulatory physiology with particular reference to regulation of energy exchange and water exchange and control of food and water intake. The changes in and inter-relationships between these have been studied in normal rats and during the cachexia that develops with growth of a tumor. The partition of energy expenditure in relation to total motor activity has been completed for the Walker tumor and has been extended to two other transplantable tumors. The effect of sodium depletion on the development of cachexia has been thoroughly re-examined in the light of this new approach and no evidence has been found to support the view that sodium depletion is an important contributor. The effect on the development of cachexia of imposed motor work coupled, variously, to feeding or drinking is being examined. Preliminary work has been done on change on excretion of anorexigenic factor in urine of tumor-bearers as compared with normal rats. A systematic examination has been started of the changes during tumor growth in response to stimuli known to modify food intake of normal animals. Alterations in water exchange during tumor growth have been analyzed and defined and work is in progress on the extent to which these changes are physiologically functional.

Radiation biology. The primary objectives of these studies are understanding the nature and duration of the chain of events resulting from sublethal irradiation or other treatments and useful application to therapy in tumor-bearing animals. When vinblastine (VLB) followed radiation within a few minutes, at a time when mitotic activity in the crypts is very low, most of the animals survived. When VLB was given 1 to 3 days after radiation, mitotic activity in the crypts fell to levels as low as those seen with 1200 rads alone, and most of the mice died in 3 to 5 days. This did not occur when the gut was shielded from the radiation. In applying these findings to therapy, the critical question is whether tumor cells and tissues essential to life of the animal have the same survival characteristics. In untreated mice bearing lymphosarcoma 1798 there were no survivors with 10^7 cells inoculated; median survival times for 7 groups of 10 mice were 14-15 days. Experimental groups were treated 3 or 5 days after inoculation, the radiation dose being 400 or 500 rads and the VLB dose 3 or 5 mg/kg. With radiation alone 12.5% of 48 mice, and with VLB alone none of 19 mice, survived. With VLB given a few minutes after irradiation 61% of 59 mice survived. This result is approximately the same as obtained by reducing the inoculum from 10^7 cells to 10^2 cells. By giving VLB shortly after irradiation, it was possible to destroy the tumor while avoiding death of the host, which would result from intestinal damage if VLB were given 1-3 days later. The excessive vulnerability to VLB is associated with processes involved in recovery from the sublethal radiation effects.

Immunogenetic factors affecting bone marrow transplantation in irradiated mice. Functional hematopoietic systems derived from bone marrow grafts are regularly obtainable but only in genetically controlled systems. Two factors demonstrated to affect the capacity of grafted marrow to perform its multiple functions are contact of marrow cells with antigens prior to inoculation, or contact with antigens of the recipient after inoculation. The marrow transplantation system is used in studying the antigen recognition system for testing both the ability of immunologically competent cells to recognize antigens and the ability of immunologically inert cells to be recognized as antigenic. Thus, antigenicity (or immunogenicity) was demonstrated to fall in naturally-occurring ranks showing degrees of difference in expression of antigens with the determinants derived from the male genome having a reduced expression. Since this reduced antigenicity produced during embryonic development of a hybrid was sufficient to produce an immunity that would interfere with growth of adult paternal tissue, but not adult tissue of the fetal genotype, it was concluded that this immunologic mechanism for controlling antigenicity evolved through the survival pressures that prevented the rejection of the hybrid fetuses as allografts. The immunologic function of grafted marrow was effectively controlled by selection of the least antigenic hybrid recipient, thus using the naturally-occurring controlled response produced by reduced antigenicity, in addition to experimentally controlling the ability of the marrow stem cells to recognize specific antigens after brief contact immediately prior to inoculation. It was thus possible to inhibit a graft-versus-host reaction without resorting to a prolonged use of drug-induced immunosuppression that may have undesirable side effects.

Radiation chemistry. We have continued studies on the effects of ionizing and ultraviolet radiation on proteins and nucleic acids and their constituents. Radiation-induced hydrogen transfer from exchangeable positions (those hydrogens attached to nitrogen or oxygen) to carbon-bound sites has been investigated for DNA, proteins and their constituents. For proteins and for purine and pyrimidine bases, nucleosides and nucleotides and deoxyribose, the G values for hydrogen transfer (number of hydrogen atoms transferred per 100 ev absorbed) are approximately equal to the G values for production of stable secondary free radicals as measured by Electron Spin Resonance. However, while secondary radical production is linear at low doses and saturates at high doses, the radiation-induced hydrogen transfer is linear in the high dose region for all compounds investigated, substantiating the theory that saturation of secondary radicals is due to an equilibrium between radical formation and radiation-induced destruction. The effects of temperature and of scavengers for electrons and neutral free radicals on the hydrogen transfer were investigated. This transfer appears to occur to a small extent due to (a) reactions of ionic radical precursors, (b) exchange processes of secondary radicals and, to a larger extent, (c) during the transformation of primary neutral radicals to secondary radicals, and (d) possibly during the radiation-induced destruction of secondary radicals. The distribution of free radicals in ultraviolet (254 nm) irradiated proteins, as determined by the free radical interceptor technique, differs markedly from those obtained previously by exposure to gamma radiation, hydrogen atoms and electrical discharge. The low free radical content of the aromatic residues indicates the importance of energy transfer processes.

Serial No. NCI-905
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Iodide-Concentrating Mechanism of the Thyroid Gland

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. Lois W. Tice, Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, NIH, Bethesda, Md. (Serial No. NIAMD, LEP-10)

Man Years

Total:	1.5
Professional:	1.0
Others:	0.5

Project Description:

Objectives: To learn about (a) the mechanism by which the thyroid gland maintains a concentration of iodide elevated above that in the blood, and (b) the mechanism by which the thyroid gland accumulates protein-bound iodine.

Major Findings:

Ultrastructural localization of thyroperoxidase in the thyroid follicle. Iodination of thyroglobulin appears to occur in the lumen of the thyroid follicle, the site of almost all the thyroid iodoprotein. A thyroperoxidase is known to be able to iodinate thyroglobulin and to effect the synthesis of thyroxine from diiodotyrosine in peptide linkage. Using cytochemical methods thyroperoxidase has now been observed on the apical plasma membrane where it can come in contact with its stored substrate and make possible the extracellular synthesis of thyroid hormone. Most of the thyroperoxidase is intracellular, however, in organelles which have to do with the synthesis and the transport of the enzyme to the apical plasma membrane.

Kinetics of iodine turnover in individual thyroid follicles. A theoretical anlaysis has been made of experimental data obtained on the uptake and release of iodine by individual follicles. There appear to be two pools of iodine in each follicle, one of which turns over rapidly, the other slowly. Uptake and release of rapidly turning over iodine is proportional to the surface area of the follicular lumen. The quantitative

properties of the follicle are determined by the number of cells per follicle. The slow pool of iodine amounts to approximately 30% of the thyroid iodine and turns over at a rate proportional to the volume of the follicular lumen. In addition, uptake and release of iodine by peripheral follicles as a class is approximately half that of central follicles.

Significance to Bio-medical Research and the Program of the Institute: Ultra-structural studies of this kind may allow a more detailed understanding of the mechanism of formation and secretion of thyroid hormone than heretofore possible.

Proposed Course of Project: To be continued.

Honors

Elected First Vice-President American Thyroid Association 1971
Board of Directors, NIH Foundation for Advanced Education in the Sciences

By invitation: Visiting Scientist, Laboratory of Nuclear Medicine, Free University of Brussels, June to August 1971

Publications:

Loewenstein, J. E. and Wollman, S. H.: Mechanisms for abnormally slow release of some thyroid radioiodine; an autoradiographic study. Endocrinology 87: 143-150, 1970.

Nève, P., Eagleton, G. B. and Wollman, S. H.: Fine structure of colloid cells in the thyroid gland. J. Clin. Endocr. and Metab. 31: 38-42, 1970.

Nève, P. and Wollman, S. H.: Fine ultrastructure of the thyroid gland of the cream hamster. Anat. Rec., in press.

Wollman, S. H.: Histologic techniques in investigation of the thyroid. In Berson, S., et al. (Eds.): Methods in investigative and diagnostic endocrinology. Amsterdam, North Holland Publishing Co., in press.

Serial No. NCI-906
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Production and Properties of Transplantable Thyroid Tumors

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Man Years

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring in the thyroid during growth, hyperplasia and involution; histologic types of thyroid tumors and their progression, and the correlation of histology with the biochemistry and physiology of the tumors.

Major Findings: None

Proposed Course of Project: To be continued.

Honors

Invited lecture. Laurentian Hormone Conference: Ultimobranchial follicles in thyroid glands of rats and mice.

Publication:

Wollman, S. H. and Nève, P.: Ultimobranchial follicles in thyroid glands of rats and mice. Recent Progr. in Hormone Res. 27: 213-234, 1971.

Serial No. NCI-928
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Intracellular Colloid Droplets

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Man Years

Total:	0
Professional:	0
Others:	0

Project Description:

Objectives: To study the properties of intracellular colloid droplets in the thyroid gland.

Major Findings: None

Significance to Bio-medical Research and the Program of the Institute: This work may lead to a better understanding of the mechanism of hormone secretion and, hence, radioisotope release in the thyroid gland.

Proposed Course of Project: To be continued.

Serial No. NCI-919
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Urinary Excretion Patterns in Leukemia

Previous Serial Number: Same

Principal Investigator: James C. Reid

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: This program is a developmental effort to expand the scope of urinary analysis. Its objectives are the development of a methodology to broaden greatly the chemical information which can be extracted from the urine and the evaluation of such information for potential utility from both the clinical and research standpoints. Experimentally, a search is being conducted for significant abnormalities in the excretion patterns of all detectable ultraviolet-absorbing components of a set of urine specimens collected from leukemic and control subjects. Such components include various nucleic acid congeners but are not limited thereto.

Methodology: The basic concept is that it may be feasible to construct an analytical system powerful enough to deal with the complexity of urinary composition by the application of a computational technique for multicomponent mixture analysis to urinary fractions obtained after preliminary chromatographic simplification. The data required are simply absorbance spectra, which can be obtained by a single non-specific instrumental procedure. The necessity for the application of many chemical procedures, each specific for a particular component, is eliminated thereby and the attendant reduction of labor would make it feasible to study many components simultaneously, i.e. to study patterns of excretion. Moreover, techniques which have been developed in this program permit quantitative estimation of the components without the necessity for chemical identification, a feature which facilitates further the recognition of pattern abnormalities if they should occur.

The preliminary chromatographic simplification is effected by a system of ion-exchange column procedures developed earlier in the course of this program. The system reduces each urine specimen to approximately fifty

fractions. Each fraction is a mixture whose components typically number about a half-dozen, and in general the individual components can be obtained in small amounts by paper chromatography. The absorbance spectra of the isolated components then serve as the basis for computing the composition of the corresponding fraction obtained from other urinary specimens.

Status: 1. During the current period work has focussed on the standardization of the computational technique, i.e., the determination of the optimal mathematical formulation, the definition of its limits of applicability, and the development of technique for checking the validity of computed solutions.

A principal problem in this connection is that of finding the best set of wavelengths for the conduct of a particular computation. The choice would be immaterial in a perfect system, but is not so when operating on experimental data. Investigation of various approaches has yielded a formulation which appears to be logically satisfactory, based upon maximization of matrix determinants as the selection criterion. Its application to successive components affords an iterative scheme designed to give a best value for each. Bordering technique is employed to avoid what would otherwise be an infeasibly long computation.

2. A second mathematical problem is that of distributing residual error, especially in situations where matrix characteristics preclude a meaningful computation for some components but not necessarily for others. No rigorous logic exists for doing this, but a heuristic approach has been formulated, based on the relative stability of matrix axes, which appears to be useful in preliminary tests.

3. Of peripheral interest, some software has been written for the 1620 plotter which augments considerably the facilities of the basic system. This would be useful to others who may wish to produce more complex computer-drawn diagrams.

Significance to Bio-medical Research and the Program of the Institute: One of the physician's major sources of information concerning the clinical status of a patient is urinalysis. Viewed as an information file, the content of urine is very great, yet comparatively little of it is available routinely at present. This project is an attempt to make more of it available and to evaluate its potential utility as a clinical aid.

In addition to the clinical aspect, such information is potentially useful in the research context for understanding the action of antileukemic drugs and the nature of the disease. Because of the generality of the methodology however, potential applications are not confined to the cancer area. In particular, it may facilitate the study of genetic variations.

One of the elements of the methodology, namely multicomponent spectral analysis, has broad potentials in analytical chemistry, where it can be applied to data from many types of spectrometers. It may simplify and speed

up some analytical procedures by reducing the necessity for preliminary isolation steps. Also, it may be useful in special applications such as the investigation of fast reactions where conventional methods cannot be applied.

Proposed Course of Project: Programming of the computational procedures described above is still in preliminary form. During the coming period they will be incorporated into a comprehensive analytical system and subjected to systematic testing.

Serial No. NCI-925
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein Synthesis and Its Control in Normal and Tumor Cells

Previous Serial Number: Same

Principal Investigator: Marco Rabinovitz

Other Investigators: Drs. Martin Gross and Satoshi Mizuno
Also studies in collaboration with Drs. T. R. Breitman and C. R. Maxwell

Man Years:

Total:	6.5
Professional:	4.0
Others:	2.5

Project Description:

Objectives:

a) To study, in both normal and tumor cells the controls exercised by these cells in regulating the synthesis of protein. This is generally made through an analysis of the biochemical lesions introduced within the pathway by deficiencies of essential nutrients or by inhibitors.

b) Where applicable, to extend the information obtained in the first objective to the tumor-bearing animal under controlled nutritional conditions, with the view of specific inhibition of protein synthesis by cancer cells or to other selective lethal effects.

Methods Employed:

a) Organic synthetic techniques are developed for preparation of potentially effective amino acid antagonists.

b) Incorporation of carbon-14 or tritium labeled amino acids into protein of isolated cells is measured. Such studies involve radioactive counting techniques and stripping film radioautography. Respiration and anaerobic glycolysis are followed by manometric techniques.

c) Subcellular components are isolated by differential and zonal centrifugation. These components are further fractionated by extraction, hydrolysis or chromatographic techniques. The characteristics of the block in amino acid utilization within these fractions are evaluated.

Major Findings:

1. Formation of an Inhibitor of Globin Synthesis in Reticulocyte Lysates (in collaboration with Dr. Martin Gross)

An inhibitor of globin chain initiation is formed by incubation of reticulocyte lysates, and this is blocked by low concentrations of hemin. The inhibitor-forming system is localized in the ribosome-free supernatant fraction, and the inhibitor, which is protein in nature, is made from available precursor in the absence of protein synthesis. Both the inhibitor and the inhibitor-forming system can be isolated from the supernatant fraction of reticulocytes by gel filtration, and both behave as high molecular weight complexes of the order of 200,000. There appears to be no low molecular weight component essential for the transformation. Our principal goal in this area is to fractionate the macromolecular components of the inhibitor-forming system in order to elucidate the biochemical steps in inhibitor formation.

Globin can be formed in the absence of hemin at lower temperatures where inhibitor formation is markedly retarded. Such globin is not converted to inhibitor when the temperature is raised, suggesting that the inhibitor is not made by a direct modification of globin chains.

2. Mechanism of Action of the Inhibitor (in collaboration with Dr. Satoshi Mizuno)

A fraction of a potassium chloride solution extract of reticulocyte ribosomes which is required for globin chain initiation can partially protect lysates against the action of the inhibitor, and it currently appears that the inhibitor acts as an antagonist for this factor. There is a delay in the onset of inhibition (2.5 min) and this is correlated with a delay in the breakdown in the polyribosome profile of the lysate. This lag can be reduced by half by preincubation of the lysate with inhibitor. Such a lag would be expected if a protein, the inhibitor, must displace an integrated factor to cause inhibition.

Analysis of polyribosome profiles of lysates during the onset of inhibition indicates that the inhibitor does not selectively block association of the larger ribosomal subunit with the messenger RNA-small ribosomal subunit complex. Our principal goal in this area is to define the exact site of inhibitor action among the several steps in globin chain initiation.

The inhibitory supernatant fraction formed in the absence of hemin also inhibits the synthesis of minor non-globin proteins in reticulocyte lysates. The significance of this observation to the mechanism of the

control of globin synthesis by hemin is not clear.

Significance to Bio-medical Research and the Program of the Institute: These studies are designed to help explain the nature of the lesions caused by nutritional deficiencies and antagonisms at the level of macromolecular biosynthesis. Such evaluations should provide a basis for our understanding of control of protein-forming systems and cell types.

Proposed Course of Project: Those areas which appear most promising will be investigated.

Publications:

Rabinovitz, M.: Some recent advances in hemoglobin biosynthesis. In Sunderman, F. W. (Ed.): Applied Seminar in Chemical Hematology. In press.

Freedman, M. L. and Rabinovitz, M.: Structure of the large β-chain polyribosome from O-methylthreonine-induced isoleucine deficient rabbit reticulocytes. Mol. Pharmacol., in press.

Maxwell, C. R., Kamper, C. S. and Rabinovitz, M.: Hemin control of globin synthesis: An assay for the inhibitor formed in the absence of hemin and some characteristics of its formation. J. Mol. Biol., in press.

Serial No. NCI-946
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Role of Hemin in the Control of Globin Synthesis

Previous Serial Number: Same

Principal Investigator: Charles R. Maxwell

Other Investigator: Marco Rabinovitz

Man Years:

Total: 3
Professional: 2
Others: 1

Project Description:

Objectives: This investigation is to determine the mechanism by which hemin stimulates in vitro synthesis of globin in a cell-free system prepared from rabbit reticulocytes.

Major Findings:

The major findings reported last year concerning the formation and general characteristics of an hemin-controlled inhibitor of protein synthesis have been polished up and a manuscript describing this work has been accepted by the Journal of Molecular Biology and should appear soon. See last year's report for details.

The major effort this year has been directed towards the isolation and purification of the inhibitor. By the use of combinations of acid precipitation and resolution, ion exchange column chromatography and gel filtration the specific activity (units of inhibitor/mg protein) has been increased by a factor of fifty with an overall recovery of 25%. The specific activity has been further increased by another factor of ten with the use of electrofocusing equipment, but the loss of material (over 90%) in this single step is prohibitively high and other methods will be investigated.

Significance to Bio-medical Research and the Program of the Institute: The knowledge of the basic control mechanism in any cell is of potential basic importance to cancer research when one considers that one simple-minded way of looking at a cancer cell is that it is a cell with a disfunction in its normal control mechanism.

Proposed Course of Project: We plan to continue this work with the original efforts being concentrated on isolation and purification of the inhibitor so that more precise measurements may be made as to its mode of operation and the evaluation of its biological importance.

Publications:

Maxwell, C. R., Kamper, C. S., and Rabinovitz, M.: Hemin control of globin synthesis: An assay for the inhibitor formed in the absence of hemin and some characteristics of its formation. J. Mol. Biol., in press.

Serial No. NCI-944(a)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Total Metabolism

Previous Serial Number: Same

Principal Investigator: Seoras D. Morrison

Man Years:

Total:	5
Professional:	2
Others:	3

Project Description:

Objectives:

(a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth and to finding the nature of the loads imposed by a tumor on the host.

(b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth.

(c) Examination of possible experimental methods of simulating in non-tumor-bearing rats the cachexia and decline of food intake found during neoplastic growth.

(d) Construction of a model of the growth of the Walker 256 tumor.

Methods Employed: The methods of indirect calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Ballistic methods of recording and partitioning total motor activity. Computer methods of numerical analysis of serial records of tumor growth and of continuous records of gaseous exchange. Sprague-Dawley rats and Buffalo rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma, but other transplantable tumors are also used.

Major Findings:

(a) The multiple compartment system of energy expenditure during tumor growth, including a compartment attributable to tumor load on the host, has been established and analyzed for the Walker tumor (Cancer Res. 31: 98-107, 1971). Extension of this work to other transplantable tumors to examine how general the phenomenon is, is being continued.

This principle has been applied to the apparent restoration of food intake after Na loading of the host. It was found that Na only rarely restores food intake but when it does it also restores the activity compartment (paper in preparation).

Further work on ballistic methods of quantitatively recording and partitioning activity [NCI-944(a), 1969] is awaiting acquisition of a suitable recording system.

A systematic examination has been started of the quantitative changes, induced by a tumor, in the response to stimuli known to modify food intake of normal animals. The Walker tumor severely depresses the response to bulk dilution of food.

(b) The sustained or increased water intake of tumor-bearing rats has been defined and analyzed with respect to the changes in all the individual compartments of water exchange during tumor growth (J. Nat. Cancer Inst. 46: 825-830, 1971).

Initial work has been completed on the extent to which increased water intake during tumor growth can be modified by coupling a work requirement to drinking. The increase is eliminated by minimal coupled work (paper in preparation). Work on this topic is continuing.

(c) Preliminary results on perfusion of normal rats with tumor-equivalent amounts of lactic acid show that this depresses food intake. This work is continuing and is being extended to include tumor-bearing rats at an early stage of tumor growth.

The anorexigenic material reported to be excreted in rat urine (e.g., Stevenson et al., 1964) is being investigated to find whether it is involved in the decline of food intake in cancer. Difficulty is being found in detecting it normally in the strain of rats used here.

(d) Experimental work on measurement and prediction of metabolizing mass of a growing solid tumor [NCI-944(a), 1968] is continuing.

Significance to Bio-medical Research and the Program of the Institute: The findings on partition of energy expenditure and of tissue compartments and on processes influencing food and water intake make it possible to examine some features of tumor-host relationships which have not previously been subject to analysis. It is believed that this may throw useful light

on the reasons for the rapid and extreme debilitation of the host during tumor growth.

Proposed Course of Project: Work on energy and water exchange of tumor-bearers will be continued with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth.

Publications:

Morrison, S. D.: Partition of energy expenditure between host and tumor. Cancer Res. 31: 98-107, 1971.

Morrison, S. D.: Water intake and exchange and hydration of rats during growth of Walker tumor. J. Nat. Cancer Inst. 46: 825-830, 1971.

Serial No. NCI-944(b)
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mathematical Programming

Previous Serial Number: Same

Principal Investigator: Florence K. Millar

Man Years

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: To facilitate the investigation of bio-medical research problems through the application of computer programming and computer data processing. Some of the projects reported here are collaborative efforts with investigators in other units of NCI and NIH.

Methods Employed: The IBM-1620 facility of the NCI has been described in previous reports [see NCI-944(b), 1969]. For some computer applications, use has been made of the central NIH computer facility (Computation and Data Processing Branch, DCRT).

Major Findings:

Computer Operations:

The 1620 has been operated as in the past on a one-shift basis with prime time shared by Drs. Reid and Millar and with use by other investigators (see below). Computer operations include routine procurement of supplies, scheduling of user time and maintenance, and making additions to the system software as needed.

During the past year there has been increasingly heavy use of the CalComp plotter attachment for on-line plotting of data. Programs have been created, or old programs modified, to provide flexibility in the use of this facility by scientific investigators for handling their results. Preliminary attempts have been made to adapt a limited three-dimensional plotting program obtained from DCRT for use on the 1620.

Computer Applications:

(1) Processing of raw data from liquid scintillation spectrometers.

The 1620 is used to reduce and further process the raw data output from double label experiments and to plot the results in a variety of bio-chemical studies as follows:

Replication of DNA as nucleoprotein (Dr. J. Shack, Mrs. B. S. Bynum, C/LPHY)

Initiation of immunoglobulin synthesis in plasma cell tumors (Dr. D. Ein, C/I)

Activation of the immune lymphocyte by antigens (Dr. S. A. Rosenberg, Dr. R. Levy, C/I)

Effect of steroids on tRNA synthesis in HTC cells (Dr. E. I. B. Thompson, C/LB)

Distribution of rare nucleosides in tRNA (Dr. A. Peterkofsky, Dr. D. M. Powers, H/LBG)

(2) Statistical analysis and curve fitting.

Standard statistical methods, including simple linear and multiple regression analysis, have been applied. In some studies of enzyme kinetics, use is made of programs developed by Cleland (Nature 198: 463, 1963) to handle the non-linearity of the reaction equations.

Studies for which the computer has application in the general area of statistics and curve fitting are as follows:

Relationships among components of material exchange measured during tumor growth (Dr. S. D. Morrison, C/LPHY)

Radiation-induced hydrogen transfer in biological macromolecules (Dr. T. C. Smitherman, Mr. H. M. Moss, C/LPHY)

Measurement of cyclic AMP levels in tissues (Dr. A. Peterkofsky, Miss C. Gazdar, Miss E. Cutler, H/LBG; Dr. J. S. Stoff, H/LKEM)

Kinetic analysis of enzymes involved in catecholamine biosynthesis (Dr. R. M. Weinshilboum, Dr. J. T. Coyle, M/LCS)

Kinetic studies on UMP-CMP kinase (Dr. E. P. Anderson, Dr. T. Q. Garvey, C/LB)

Multiple regression program testing (Mr. D. E. Towne, CH/SINFC)

Significance to Bio-Medical Research and the Program of the Institute: Mathematical and computer programming applications contribute to the investigation of research problems by providing (1) accurate and efficient methods for handling laborious calculations of data and visualizing results, (2) the ability to store and retrieve large amounts of data, and (3) opportunities for testing mathematical models based on experimental results.

Proposed Course of Study: Computational techniques and programs will continue to be developed and applied in response to computer-related needs of the scientific staff.

Serial No. NCI-916
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Characterization and Comparison of the Nucleic Acids, Nucleoproteins and Related Components of Normal and Malignant Tissues

Previous Serial Number: Same

Principal Investigator: Joseph Shack

Other Investigator: Barbara S. Bynum

Man Years:

Total:	2.0
Professional:	2.0
Others:	0

Project Description:

Objectives: The major objective of the past year has been the further elucidation of the process of replication of DNA in dividing HeLa cells. Specifically, the principal objective has been to 1) determine the physical and chemical basis for certain differences we have observed to exist between deoxynucleoprotein particles containing newly-formed DNA and the previously existing (old) nucleoprotein, and 2) further development of procedures for physical separation and purification of the newly-formed nucleoprotein.

Major Findings:

1) The electrophoretic mobility of newly-formed nucleoprotein is somewhat lower than that of the "old" nucleoprotein, a fact which has proved useful in partial separation. After denaturation of the DNA component or partial removal of protein by appropriate methods of extraction the patterns coincide.

2) Results of chromatography on hydroxyapatite show that the newly synthesized DNA of nucleoprotein particles is more resistant to denaturation than the "old" DNA. After removal of part of the protein the difference is no longer observed.

3) Previously reported differences between sedimentation properties of "new" and "old" nucleoprotein disappear on removal of part of the protein.

4) We conclude that some of the unique differences between newly-formed and old nucleoprotein are due to differences of configuration brought about by a class of easily dissociated protein which may include various enzymes involved in replication.

Significance to Bio-medical Research and the Program of the Institute: To the extent that cancer is a problem of cellular growth, the content of this project is probably relevant to the cancer problem.

Proposed Course of Project: In the immediate future: logical and feasible extensions of what was described above. One of us (J. S.) plans to terminate his participation in the project during the early part of the next fiscal year.

Serial No. NCI-945
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of the Factors Regulating the Initiation of Specific Protein Synthesis During Embryonic Development

Previous Serial Number: Same

Principal Investigator: Beverly Peterkofsky

Other Investigator: Robert Diegelmann, PHS Fellow, Guest Worker

Man Years:

Total:	4.5
Professional:	3
Others:	1.5

Project Description:

Objectives: To investigate the factors controlling the activity of the enzyme, tyrosine aminotransferase, and controlling the synthesis of the structural protein, collagen, during embryonic development.

Major Findings:

A. Tyrosine aminotransferase

During the purification of chick embryo tyrosine aminotransferase, it was observed that the activity was quite unstable when crude extracts were stored at 4°, while the activity in heat-treated or DEAE cellulose fractionated extracts was stable. In view of current interest in the mechanisms by which proteins are degraded or inactivated in animal cells, it was decided to investigate this observation further.

It was found that the protein fraction which was not adsorbed onto DEAE cellulose at pH 7.6 could inactivate partially purified, stable TAT. Inactivation was measured by incubating partially purified TAT with the unadsorbed protein fraction. TAT activity was measured before and after the incubation. Three different pH optima were found for the inactivating activity (4.1, 5.2 and 9), suggesting that several different proteins were involved. The inactivation at both pHs 4.1 and 5.2 was inhibited by pyridoxal phosphate, the cofactor for aminotransferase enzymes, but with time the inhibition was reversed suggesting that pyridoxal phosphate was being metabolized by an

enzyme present in the unadsorbed protein fraction. Since the dephosphorylation of pyridoxal phosphate by acid phosphatase has been reported, the unadsorbed protein fraction was assayed for and found to contain acid phosphatase activity. The possibility that this enzyme was responsible not only for dephosphorylation but also for the inactivation of TAT was also investigated. The following observations suggest that acid phosphatase is responsible for the inactivation of chick TAT at pH 4.1 and 5.2.

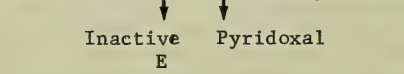
1) Inactivating ability and phosphatase activity of the unadsorbed protein fraction are both labile upon heating at 50° for 5 minutes.

2) Inhibitors of acid phosphatase activity against p-ninophenyl phosphate also prevent the inactivation of TAT by the unadsorbed protein fraction. These compounds include NaF, ATP and pyridoxal phosphate.

3) Inactivating activity and phosphatase activity both appear to be lysosomal.

Furthermore, commercially purified preparations of wheat germ and phosphatase, chicken intestine alkaline phosphatase and E. coli alkaline phosphatase also inactivate chick TAT.

The mechanism by which this inactivation occurs has been deduced from experiments with rat liver TAT and purified pig heart aspartic aminotransferase. Rat liver TAT is not inactivated but is converted into apoenzyme by wheat germ acid phosphatase. It is inactivated by alkaline phosphatase. Aspartic aminotransferase which contains tightly bound pyridoxal phosphate is not affected in any way by either the animal or bacterial alkaline phosphatases. The tentative outline of the mechanism of inactivation of chick TAT is as follows:



In our previous studies we had shown that chick TAT apoenzyme was extremely labile.

B. Collagen

1. Isolated tissues labeled in vivo.

Further studies were carried out on the relative rate of collagen synthesis in chick embryos at various stages of development. The study was expanded to isolated connective tissues. Embryonated eggs were injected with proline-^{14}C and after 1 1/2- 3 hours of incubation skin, lower limbs and frontal bones

were removed. Homogenates of the tissues were prepared, treated with ribonuclease to remove aminoacyl-tRNA and precipated with trichloroacetic acid to isolate radioactive protein. Radioactivity in collagen (C) and noncollagen protein (NCP) was measured after treatment with purified collagenase as described in our previous report. Results were expressed as the ratio of radioactivity in collagen compared to noncollagen protein (C/NCP) or were converted to the relative percent collagen synthesized by the following equation:

$$\text{relative percent collagen} = \frac{CPM_C}{(CPM_{NCP} \times 5.4) + CPM_C} \times 100$$

Since collagen contains 5.4 times more imino acids than an average protein, this value was applied as a correction factor.

It was found that the relative synthesis of collagen in skin remained constant while it increased markedly in those tissues where bone development was occurring, such as the lower limb, which is made up in large part by the tibia, and the frontal bone. The most significant findings during the 8th and 16th day of embryonic development may be summarized as follows:

a) Skin: At the eighth day, the relative amount of collagen synthesized was approximately 3%. This value increased only slightly and varied between 4-5% during the entire period.

b) Lower limb: At the eighth day, the relative amount of collagen synthesized was about the same as in skin, 5%, but it increased constantly until a maximum of 28% was reached by the 14th day.

c) Frontal bone: The earliest point at which this tissue could be removed for analysis was at 11 days. The relative amount of collagen synthesized at that point was 27% but it increased rapidly in several days to a maximum of 60% at 15 days.

These results suggested that during formation of bone tissue, collagen was being selectively synthesized. This could be explained by two alternative situations: 1) an overgrowth of collagen-producing cells at the expense of other cell types; 2) differentiation of one cell type producing a low level of collagen to another type producing a high level.

Bone tissue changes during embryonic development from a cartilagenous type, in which chondroblasts produce mucopolysaccharide and collagen, to ossified bone, in which osteoblasts produce mineral matrix and collagen. Since osteoblasts contain a very high level of the enzyme alkaline phosphatase, while chondroblasts and other types of cells contain negligible levels, the three tissue types were analyzed for the presence of this enzyme and the results can be summarized as follows:

a) Skin: The specific activity was the lowest of all the tissues (30 mμmoles/

min/mg protein) and constant during the period from 8-18 days of embryonic development.

b) Lower limb: At 8 days the specific activity was about the same as in skin but increased rapidly after 13 days to reach a level of 600 mμmoles/min/mg at 18 days.

c) Frontal bone: At 11 days the specific activity was already quite high, 1500 mμmoles/min/mg, and increased further to reach a maximum of 2300 by 14-16 days.

These results suggest that during the period when the population of cells in bone tissue is changing from one predominantly chondroblastic to one predominantly osteoblastic, the ability to synthesize collagen increases.

Since the level of a protein in animal cells is dependent on a balance between synthesis and degradation, the possibility existed that the changes in the ratio C/NCP increased because of an increase in the degradation of NCP with the concommitant redistribution of radioisotope into C, resulting in a higher ratio. Experiments in which embryos were pulse labeled and C and NCP analyzed for several days afterwards, showed that there were no significant changes in C/NCP, so that the increased ratios observed are in fact due to an increase in the relative rate of collagen synthesis.

2. Isolated tissues labeled in vitro in organ culture.

Tissues were removed from embryos at 13 and 15 day stages of development and incubated for 1 1/2 hours in Earle's balanced salt solution plus proline-^{14}C. The tissues were then washed and analyzed as above. Although the relative amount of collagen synthesized was highest in the frontal bone as compared to skin and lower limb, it was considerably lower than observed after in vivo labeling; 20% in vitro compared to 60% in vivo. These results suggest that humoral factors may be involved in maintaining bone cells in their highly differentiated state.

3. Collagen synthesis in tissue culture.

The assay for C and NCP has been utilized to study relative collagen synthesis during the growth cycle of various fibroblast cultures. Previous determinations of collagen synthesis in cell culture have only been able to measure fully hydroxylated collagen since the methods depended on measuring hydroxyproline-^{14}C derived from the precursor proline-^{14}C. Our assay allows measurement of collagen whether hydroxylated or not. The studies were conducted on two established lines, hamster fibroblasts and mouse L-cell fibroblasts, and primary cultures of chick embryo skin fibroblasts. The relative synthesis of collagen was found to be constant throughout growth in L cells and embryo fibroblasts. No synthesis was detected in hamster fibroblasts.

Significance to Bio-medical Research and the Program of the Institute: These projects are involved in elucidating mechanisms which regulate levels of specific proteins within a cell and those which control the alteration of one cell type into another. The knowledge of such normal mechanisms of regulation will be a prerequisite for understanding the defects of regulation which cause cells to become neoplastic.

Proposed Course of Research:

1. Tyrosine aminotransferase

A. Further characterization of the enzymes in embryo liver responsible for inactivation of TAT. Although the activities at pH 4.1 and 5.2 have been identified as acid phosphatases on the basis of inhibition studies, it is known that there are several different acid phosphatases present based on gel electrophoresis patterns. Liver extracts will be fractionated by column chromatography to determine whether all acid phosphatases can inactivate TAT or whether there are differences in specificity. The factor responsible for inactivation at pH 9 will be characterized since it has not been positively identified as a phosphatase.

B. Investigation of the role of phosphatases in the turnover of TAT in embryo liver cells. We have succeeded in growing primary cultures of embryonic liver cells which contain TAT. After glucorticoid steroid induction of TAT, removal of the steroid results in cessation of induced synthesis and a rapid lowering of the level of enzyme because of enzyme degradation. With such a system, the effect of various substances on the degradation can be tested. Compounds known to inhibit or repress the synthesis of acid or alkaline phosphatase will be tested.

2. Collagen

A. Correlation between increased collagen synthesis and ossification. Further evidence will be sought to support the conclusion that osteoblasts are more fully differentiated with respect to collagen synthesis. Calcium-45 uptake by lower limb and frontal bone of embryos at various stages of development will be determined. This should indicate precisely the stage at which ossification occurs.

B. Identification of factors which may influence the state of differentiation of bone cells. As mentioned above, the relative amount of collagen synthesized by frontal bone decreases when the tissue is incubated *in vitro*. Various compounds such as parathyroid hormone, thyrocalcitonin, dibutyryl cyclic AMP and extracts of bone tissue will be tested to see whether there is enhancement or further repression of collagen synthesis. Isolated cells will also be grown in tissue culture to determine whether the differentiated state can be maintained under these conditions.

C. Determination of collagen synthetic ability in a cell-free system. Cell-free systems derived from bone tissue of embryos at various stages of

development will be used to analyze whether cellular factors are responsible for altered rates of synthesis.

Publications:

DeVivo, D. C., and Peterkofsky, B.: The effect of α-ketoglutarate and other α-keto acids on the recovery of tyrosine aminotransferase from chick embryo liver. J. Biol. Chem. 245: 2737-2746, 1970.

Peterkofsky, B., and Diegelmann, R. F.: Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10: 988-994, 1971.

Serial No. NCI-948
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Control of Ribo- and Deoxyribonucleotide Synthesis

Previous Serial Number: Same

Principal Investigator: Theodore R. Breitman

Other Investigators: Lawrence R. Solomon and
collaboration with Marco Rabinovitz

Man Years:

Total:	4.5
Professional:	4.0
Others:	0.5

Project Description:

Objectives: To clarify metabolic transformations and control mechanisms of purine and pyrimidine ribo- and deoxyribonucleotide biosynthesis.

Major Findings:

A. The Metabolism of Pseudouridine

The growth on pseudouridine of two pyrimidine auxotrophs of Escherichia coli (Bu⁻ and W63-86) was markedly enhanced when glycerol replaced glucose as a carbon source or when cyclic AMP was added to medium containing glucose. These results indicated that an enzyme, catalyzing a reaction in the pathway of pseudouridine conversion to uracil, was sensitive to catabolite repression.

The following pathway was proposed for pseudouridine utilization:

(1) pseudouridine + ATP $\xrightarrow[\text{kinase}]{\text{pseudouridine}}$ pseudouridine-5'-P

(2) pseudouridine-5'-P $\xleftarrow[\text{synthetase}]{\text{pseudouridylate}}$ uracil + ribose-5-P

Pseudouridylate synthetase was sensitive to catabolite repression in strains Bu⁻ and W63-86. In contrast, strains B5RU and W5RU, mutants of Bu⁻

and W63-86, selected for their ability to grow rapidly on pseudouridine in the presence of glucose, had high levels of pseudouridylate synthetase in the presence of glucose. In the case of B5RU, but not W5RU, synthetase activity was greater in cells grown on glycerol or on glucose plus cyclic AMP than on glucose.

Pseudouridine kinase, the enzyme catalyzing reaction (1) in the proposed pathway outlined above, had not been described previously. An effort was therefore undertaken to determine this activity. Pseudouridine kinase activity was found in E. coli and, like pseudouridylate synthetase, was sensitive to catabolite repression. A parallelism between pseudouridine kinase and pseudouridylate synthetase has been observed. Strains either have both activities or neither activity. The requirements of the kinase activity have been delineated and purification of the enzyme has been undertaken.

B. Methionineless Death in Escherichia coli (in collaboration with M. Rabinovitz)

The biochemical basis for methionineless death in strains of E. coli 15, described in the 1970 Annual Report, has been clarified. The DNA degradation that was associated with this phenomenon appears to be triggered by the action of the r15 restriction enzyme. Strains of E. coli 15 that are lacking the r15 restriction phenotype neither die nor degrade their DNA in the absence of methionine.

C. DNA Degradation during Thymineless Death

A new observation has been made which may explain the long observed but still unexplained phenomenon of thymineless death. Under thymine deprivation approximately 20% of the DNA of all thymine auxotrophs that have been studied is degraded. This degradation is random, affecting all regions of the chromosome. It therefore appears likely that errors in repair when thymine is re-introduced into the medium results in the loss of viability.

Significance to Bio-medical Research and the Program of the Institute: Findings pertaining to an elucidation of mechanisms involved in the metabolism of nucleic acid components are important as they may suggest possible means of therapeutic control of cellular growth.

An elucidation of the methionineless and thymineless death phenomena appears to be extremely important as it may lead to an approach to specifically trigger a mechanism allowing a cell to destroy itself.

Proposed Course of Project: If the necessary technical and postdoctoral help is obtained, the following projects can be worked on:

1. Purification and characterization of pseudouridine kinase and pseudouridylate synthetase.

2. Purification and characterization of the r15 gene restriction-

modification product.

3. Further clarification of the enzymatic degradation of DNA during thymine deprivation.

Publications:

Breitman, T. R.: Pseudouridylate synthetase of Escherichia coli: Correlation of its activity with utilization of pseudouridine for growth. J. Bacteriol. 103: 264-265, 1970.

Serial No. NCI-936
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Time-dependent effects of an initial treatment on tolerance to subsequent treatments; radiation and vinblastine, with an application to experimental therapy

Previous Serial Number: Same

Principal Investigator: Willie W. Smith

Man Years:

Total:	3
Professional:	2
Others:	1

Project Description:

Objectives: To obtain an understanding of the chain of events resulting from an initial sublethal treatment, particularly as related to tolerance and effectiveness of subsequent treatments.

Major Findings:

Mice given sublethal irradiation followed after given intervals by a second irradiation show a time-related response similar to that of cells in culture, although for actinomycin D as the second treatment this is not the case. In the present experiment we have studied the response to vinblastine as the second treatment. Although deaths were numerous when this was given in a normally sublethal dose 1-3 days after irradiation, very few deaths occurred when VLB was given a few minutes after irradiation or when the gut was shielded during irradiation. The deaths usually occurred about 4 days after VLB injection, and appeared to be at least partially associated with mitotic activity in the intestinal crypts. The overshoot in mitotic activity in the crypts and the toxicity of VLB tended to be maximum 3 days after irradiation, depending somewhat upon the radiation and VLB doses.

Using lymphosarcoma P-1798 we investigated a possible difference in host vs. tumor response in mice given sublethal radiation followed in a few minutes by VLB. The median survival time of mice given 10^7 cells and no treatment was 16 days, none surviving. With 4 mg VLB/kg on day 3 none survived, with 400 rads 5% survived, and with VLB a few minutes after irradiation 60% survived. With 5 mg VLB/kg on day 5 none survived, with 500 rads 25%

survived and with radiation plus VLB 90% were alive and well after 15 weeks, when the experiment was terminated. Thus for a short time after irradiation, tumor cells were more sensitive to VLB than the intestinal crypt cells. We saw no evidence that destruction of hemopoietic cells was responsible for death in these animals.

Significance to Bio-medical Research and the Program of the Institute: Agents useful in cancer therapy are generally damaging to host as well as tumor, and their effectiveness is usually limited by toxicity to the host. Our research program is directed to increased understanding of the events resulting from such agents as radiation and drugs and the time-dependent interactions of treatments given sequentially. In the experimental situation it is possible to develop a physiological basis for effective therapy within the limitations of tolerance and in so doing to acquire a greater comprehension of the essential functions of particular tissues.

Proposed Course of Project: I plan to continue studies relating to post-treatment physiological effects and their influence on tolerance to subsequent treatments.

Publications:

Smith, W. W., and Wilson, S. M.: Persistence of radiation damage: Hemopoietic effects in irradiated mice treated with actinomycin D. In Stohlman, F., Jr. (Ed.): Hemopoietic Cellular Proliferation. New York, N. Y., Grune & Stratton, Inc., 1970, pp. 49-55.

Serial No. NCI-941
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunogenetic factors affecting bone marrow transplantation in irradiated mice: Modification of the functional capacity of bone marrow

Previous Serial Number: Same

Principal Investigator: Delta E. Uphoff

Man Years:

Total:	3.5
Professional:	1.0
Others:	2.5

Project Description:

Objectives: To study factors affecting functional capacity of bone marrow transplants in irradiated mice of various genetic constitution with the ultimate goal of improving survival following marrow grafting by controlling the immunologic function of the grafted marrow without reducing the capacity of the marrow to grow.

Methods Employed: Survival studies were conducted on donor-recipient combinations with differing degrees of genetic disparity. Changes in functional capacity of grafted marrow were indicated by characteristic changes in survival patterns and indicated the ability to respond to erythropoietic stimuli, various antigenic stimuli, as well as alteration of the leukemogenic process. All facets of the multifunctional hematopoietic system were tested in this one system and included the establishment and persistence of the marrow graft and the effects upon the ability to differentiate between self, nonself, and to recognize foreignness.

Major Findings: The test system described in detail in the previous report was developed using a single incompatible donor-recipient combination. The versitility of this system was tested by continuing to use the same incompatible combination of strains while the universality of the observations was tested by using other combinations of strains with different genetic disparities.

The maternal modification of the expression of paternally derived tissue antigens controlled the severity of the graft-versus-host reaction in lethally

irradiated reciprocal hybrid mice inoculated with marrow from parental strains when the recipients were either homozygous or heterozygous for the major histocompatability locus (H-2). To most effectively control the graft-versus-host reaction the quantitative difference in antigenicity must be controlled by genetic selection. The next most effective method was to utilize the qualitative difference in antigenicity occurring naturally as a result of the maternal modification of the expression of paternally derived antigens.

The maternal modification of antigenicity was considered to be the immunologic mechanism evolved to prevent the rejection of the hybrid fetus as an allograft. To test this hypothesis, AKR mice that had previously rejected skin allografts for reciprocal hybrid genotypes or the parental strain skin were lethally irradiated and inoculated with either hybrid or parental strain marrow. It was demonstrated that an immunity produced by tissue of the fetal genotype that interfered with the growth of parental strain tissue did not interfere with the growth of adult tissue of the genotype of the hybrid fetus. By varying the antigenicity of the "priming" antigen and the antigenicity of test antigen, gradations between the two extremes were obtained, demonstrating that this immunologic mechanism alone could ensure survival of tissue of the genotype of the fetus. These experiments did not eliminate the possibility that other supplementary mechanisms may exist to provide additional survival advantages for the fetus.

In vitro preincubation of bone marrow with allogeneic erythrocytes was used to diminish the severity of the graft-versus-host reaction or to increase the reactivity of the grafted marrow. This observation was developed into the concept that antigenicity (or immunogenicity) could be ranked. Thus, a direct relationship was established between tolerance and sensitization by changing the antigenicity of the "priming" antigen in relation to the antigenicity of the recipient.

Homologous or heterologous antigens, as well as soluble and particulate antigens, were used to modify the functional capacity of compatible allogeneic marrow transplants, thus demonstrating that the permanent alteration of marrow stem cell function by exposure to antigens in vitro is apparently a general phenomenon.

Significance to Biomedical Research and the Program of the Institute: This research program has revealed naturally occurring immunogenetic phenomena which help to account for the more successful results obtained under genetically controlled experimental conditions than under clinical conditions. The modification of the reactive potential of immunologically competent cells and the induced or acquired modification in expression of tissue antigens are relevant to immunotherapy of neoplasms and to successful organ transplantation, while maternal modifications are basic to both developmental biology and mammalian genetics.

Proposed Course of Project: This project will be continued essentially in its present form since this test system appears to be the most sensitive yet developed for investigating the antigen recognition system. Attempts will be made to investigate the mechanisms by which modifications are induced. Other aspects of this project reported in previous years are being continued.

Honors and Awards:

Doris L. Crockett Distinguished Service Award from Russell Sage College.

Publications:

Uphoff, D. E.: Radiation resistance and sensitivity in mice transferred by foster nursing. European J. Clinical and Biological Res. 15: 303-304, 1970.

———: Reduced incidence of thymic lymphomas in AKR recipients of syngeneic marrow preincubated with bovine serum albumin. J. Nat. Cancer Inst. 44: 1099-1106, 1970.

———: Maternal modification of tissue antigenicity and the histocompatibility-2 (H-2) locus. J. Nat. Cancer Inst. 45: 1035-1037, 1970.

———: Maternal modification of antigenicity: An immunologic mechanism to ensure survival of mammalian fetuses. J. Nat. Cancer Inst. 45: 1189-1196, 1970.

——— and Draper, L. R.: Graft-versus-host modification by in vitro preincubation of marrow with subcellular antigens. J. Nat. Cancer Inst. 46: 1139-1141, 1971.

———: Modified antigenicity: An immunologic mechanism for species survival. Transplantation Proc. (in press).

Serial No. NCI-942
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effects of Ionizing Radiation on Macromolecules of Biological Importance

Previous Serial Number: Same

Principal Investigator: Peter Riesz

Other Investigators: T. C. Smitherman and D. Marciani

Cooperating Units: F. H. White, Jr., Laboratory of Biochemistry, NHLI, Project Serial No. NHLI-216;
H. Kon, Section on Molecular Biophysics, LPB, NIAMD, Project Serial No. NIAMD-LPB-12.

Man Years:

Total:	3.5
Professional:	3.0
Others:	0.5

Project Description:

Objectives: To study the effect of ionizing and ultraviolet radiation on biological macromolecules and their constituents. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species formed in the surrounding medium by radiation attack the DNA. In the case of water, these species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity of these macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

Methods Employed: When dry proteins or nucleic acids are irradiated with gamma rays or ultraviolet radiation in the absence of air (at 10^{-4}mm pressure), trapped free radicals are generated. Subsequent exposure to tritiated hydrogen sulfide or hydrogen iodide leads to the formation of

carbon-tritium bonds. After removal of exchangeable tritium the protein is hydrolyzed, the amino acids are separated on an automatic amino acid analyzer and the tritium content of each amino acid is determined by means of an associated flow counter. It has been shown that the tritum distribution so obtained corresponds closely to the free radical distribution at the time of exposure to the tritiated free radical interceptor.

Trapped radicals in lyophilized proteins and nucleic acids were produced by:

(a) Gamma-radiolysis using a Co^{60} source.

(b) Photolysis with 2537 Å light using a low pressure Mercury arc. A PCQ-X1 Photochemical Lamp with 4 circular lamps was used; 80-90% of the total radiation is at 2537Å.

(c) Electrical discharge was generated by a high frequency induction coil (Tesla Type) adjusted for maximum voltage (40-50 kv).

The kinetics of the reaction of free radicals in the irradiated macromolecule with various radical interceptors were followed by means of electron spin resonance spectroscopy in collaboration with Dr. Hideo Kon (Section on Molecular Biophysics, LPB, NIAMD).

Proteins and DNA were tritium labeled on exchangeable sites by exposing the dried materials to tritiated water vapor (1 curie/gm) at 20 torr for 10 minutes in a high vacuum system located in a hood in the Isotope Laboratory (Bldg. 21).

Major Findings:

1. Hydrogen Transfer in γ-Irradiated Lyophilized DNA and Its Constituents (with Dr. T. C. Smitherman).

G-values for the γ-radiation induced hydrogen transfer from exchangeable to carbon-bound sites in lyophilized bases, deoxynucleosides and deoxynucleotides, respectively, at room temperature are 0.33, 1.16, 1.89 for thymine; 0.16, 0.78, 1.45 for cytosine; 0.19, 0.38, 0.67 for adenine; (not determined), 1.41, 0.81 for guanine, and 2.66 for deoxy-D-ribose. For DNA from salmon sperm, calf thymus and chicken blood, the values are 0.27, 0.37, 0.29 and for heat-denatured salmon sperm DNA 0.34. G-values for hydrogen transfer in DNA constituents are approximately equal to the initial radical yields within the two- to three-fold experimental uncertainty of the previous ESR measurements. Although the G-values for hydrogen transfer in DNA are lower than the reported initial radical yields, the experimental uncertainty of the ESR values is considerably greater. Unlike the ESR radical yields which saturate at doses of a few megarads, hydrogen transfer is linear as a function of dose in the range of 5-30 megarads. These results are consistent with the hypothesis that (a) at dose saturation the rate of radical destruction is equal to the rate of radical formation and (b) the transfer of tritium to

carbon takes place either during the radiation-induced radical destruction or as a result of the reactions of the precursors of the secondary radicals. For DNA one of these two conditions may not be satisfied. Radicals may be destroyed by reacting with hydrogen atoms or with electrons or holes followed by proton or hydride ion transfer. In general, G-values for hydrogen transfer increase in the sequence base, nucleoside, nucleotide and pentose as previously observed for the initial radical production. Salmon sperm DNA, tritium labeled by γ-radiation induced hydrogen transfer, was enzymatically degraded and chromatographed on Dowex-1. The tritium-labeled products coeluted with the normal nucleotides and the distribution of tritium is as follows: thymidylic acid, 53%; deoxyadenylic acid, 18%; deoxyguanylic acid, 8%; and deoxycytidylic acid, 21%. It is of interest to compare these results with previously reported ESR studies of DNA which have shown that about 50% of the unpaired spins are located on thymine. However, it should not be assumed that the tritiated materials which coelute with the natural nucleotides are identical with them.

An investigation of the radiation induced hydrogen transfer in thymine has shown that the non-exchangeable tritium is distributed as follows: 35% in dihydrothymine, 58% in thymine and 7% in other products. Similar results were found at doses of 14 and 30 megarads.

These products could be explained in terms of hydrogen transfer reactions to radical species previously observed by ESR in γ-irradiated thymine monohydrate crystals. These include the 5,6-dihydro-5-thymyl and 5,6-dihydro-6-thyml radicals (precursors of tritiated dihydrothymine) and the radical formed from thymine by loss of a methyl hydrogen (the precursor of tritiated thymine).

Radiation induced hydrogen transfer in tritiated dihydrothymine leads to the following tritium distribution: 60% in dihydrothymine, 9% in thymine and 31% in other products. The G-value for hydrogen transfer (1.5) is equal to the total radical yield observed by ESR studies. The low yield of tritiated thymine shows that the intermediate radical does not undergo a radical-induced exchange process.

2. <u>The Mechanism of Radiation-Induced Hydrogen Transfer in Lyophilized Ribonuclease</u>

The radiation-induced transfer of tritium to non-exchangeable sites in RNase may occur during the radiation-induced destruction of the secondary radicals (those which have a long lifetime at room temperature in the absence of radiation) or prior to their formation. A plot of the number of secondary radicals produced at room temperature against dose is linear at low doses but saturates above 5 megarads when the rate of radical destruction by radiation is equal to the rate of radical formation. If tritium were incorporated <u>only</u> during destruction of secondary radicals (e.g. by addition of hydrogen atoms) then tritium incorporation should be non-linear in the low dose region and become linear when dose saturation of secondary radicals sets

in. Previously, tritium incorporation for RNase was found to be linear in the 5-30 megarad region, but no reliable data were available in the 0-5 megarad region, due to experimental difficulties. In recent experiments using large numbers of samples, it has been possible to show that tritium incorporation is linear in the 0-5 megarad region.

To explain this linearity, one needs a mechanism utilizing intermediates that reach a steady state, or saturation level at doses so small that they are not observable, and this requires intermediates whose disappearance processes are several orders of magnitude faster than the radiation-induced destruction of secondary radicals. These could be the ion-radicals initially produced by radiation or the primary neutral radicals, which are stable at dry ice temperature but change to the "secondary radicals" at room temperature in a time of about 10 minutes.

Nitric oxide is the classical scavenger for neutral free radicals with low reactivity towards excited states or ionic intermediates. Being itself a free radical, nitric oxide reacts very rapidly with other free radicals. Tritium transfer in RNase in the presence of 600 torr of NO was reduced to approximately 10% of its normal value. This result suggests that tritium transfer does not take place primarily as a result of the reaction of primary cationic and anionic radicals but during the conversion of the primary neutral radicals to the secondary radicals. This inference was strongly supported by recent experiments in which tritium incorporation was measured by RNase irradiated at dry ice temperature. At this temperature the ionic radicals have a very short lifetime (less than 1 sec.) and the primary neutral radicals are stable. Tritium incorporation was increased three-fold when samples previously irradiated to about 4.4 megarads were allowed to warm from dry ice to room temperature and kept at that temperature for two hours in vacuo before addition of water. This experiment indicates that a major fraction of the T incorporation occurs during conversion of neutral primary to neutral secondary radicals. This explanation is also consistent with the observation that cupric ions (2 ions/RNase) reduce the G-value for tritium incorporation to 1/3 but cadmium ions have no effect. If the change from primary to secondary neutral radicals involves the long range migration of carbon radicals by successive hydrogen atom abstraction, then cupric ions would be expected to inhibit this process. Carbon-free radicals in aqueous solutions are oxidized by cupric but not by cadmium ions.

The G-value for tritium incorporation at dry ice temperature is 0.5 (no warming up before addition of water) and 2.5 at room temperature. The tritium incorporation at dry ice temperature (G = 0.5) could be due either to destruction of the primary neutral radicals by radiation or take place as a result of reaction of the primary ionic radicals.

3. Tritium Distribution among Individual Amino Acid Residues in Lysozyme Labeled by Radiation-Induced Hydrogen Transfer (with Dr. T. C. Smitherman and Dr. F. H. White, Jr.)

Two groups of mechanisms for the R.I.H.T. process can be distinguished:

(A) those involving tritium exchange of radicals with the -NT- groups of adjacent peptide bonds, and (B) those requiring addition of free hydrogen atoms to carbon radicals. Since about 30% of the exchangeable hydrogen in lysozyme exchange very slowly, they are not labeled in the initial exposure to tritiated water vapor. If mechanism (A) applies, then those residues adjacent to internally hydrogen-bonded amides would be expected to have an unusually low specific activity. On the other hand, mechanism (B) does not predict such a result since hydrogen atoms are free to diffuse throughout the molecule at room temperature. Lysozyme labeled by the "RIHT" process, using 5 curies/ml tritiated water and irradiated to 26 megarads was chromatographed on Biorex 70. After reduction, carboxymethylation and chymotrypsin digestion, the peptides were partially separated on a phosphocellulose column, using the method of Canfield and Anfinsen. Individual peptides were then obtained by high voltage paper electrophoresis. Individual peptides are being subjected to amino acid analysis and counting. Preliminary results indicate the following:

(1) The tritium distribution by RIHT is significantly different from that obtained by addition of HST after γ-radiolysis.

(2) Seven peptides (of a total of about 19 which are now available) have been analyzed.

(3) Tritium is widely distributed throughout the lysozyme molecule and the specific activities of a given residue differ from the average specific activity of all the residues of a specified amino acid by as much as a factor of 10.

4. Distribution of Tritium between Damaged and Native Fractions in Ribonuclease Labeled by Radiation-Induced Hydrogen Transfer. Effect of Cupric Ion (with Dr. D. Marciani)

Native ribonuclease and a cupric-ribonuclease complex (4 Cu^{2+}:1 RNase) were tritium labeled by radiation-induced hydrogen transfer at a dose of 15 megarads. Chromatography on Bio-Gel P-60 revealed 3 damaged components of low enzymatic activity in addition to the native fraction in both bases. The tritium specific activities of the damaged fractions were approximately twice those of the native fractions. The presence of cupric ion reduced the tritium specific activities of all fractions by a factor of about 5. The per cent of protein in the undamaged fraction was increased from 44% to 60% in the presence of copper. These results indicate that hydrogen transfer is preferentially associated with other radiation damage, that cupric ion reduces hydrogen transfer and also protects RNase molecules against radiation damage. The association of hydrogen transfer with other radiation damage is not a consequence of two successive ionizing events occurring in the same RNase molecule. A calculation based on "target" theory shows that at a dose of 15.3 megarads, only 6% of the molecules are "hit" twice (D_{37} for ribonuclease irradiated in vacuum and dissolved in aerated water in 35 megarads.)

5. Distributions of Free Radicals among Amino Acids from Lyophilized Ultraviolet-Irradiated Proteins (with Dr. F. H. White, Jr., and Dr. H. Kon)

The effects of ultraviolet-irradiation at 254 nm upon lyophilized ribonuclease, lysozyme, insulin, and chymotrypsinogen have been investigated by electron spin resonance (ESR), enzymatic assay, and labeling of free radical sites with tritiated hydrogen sulfide (HST). The ESR signal of the irradiated protein diminishes on exposure to HST, and tritium becomes covalently bound to carbon. The distribution of tritium among the amino acids of each protein, studied as an indicator of the carbon free radical distribution, differs markedly from those observed previously to result from exposure to gamma radiation, electrical discharge, or hydrogen atoms. However, the earlier observation that the tritium distribution is influenced by protein conformation holds true as well for ultraviolet-irradiation. Moreover, the distributions of tritium among the amino acids of ultraviolet-irradiated proteins indicate a broad scattering of free radicals. Tyrosine and phenylalanine, residues that absorb light energy in the region of the wavelength employed, were not particularly important as radical carriers. Thus, for ribonuclease, these residues incorporated 3.8 and 1.5% of the total tritium, but they absorb 51 and 12% of the light, respectively. These results, together with the observed low recoveries of methionine, an amino acid that does not absorb at 254 nm, add weight to the concept that a migration of energy ensues after the initial absorption of light energy and that photolytic damage may thus be due to destruction of amino acids other than those initially absorbing the ultraviolet-radiation.

Significance to Biomedical Research and the Program of the Institute:

Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. To facilitate the understanding of the effects of ionizing radiation on cells, it is important to gain insight into the molecular damage to proteins and nucleic acids.

Proposed Course of Project: To continue studies of radiation-induced hydrogen transfer and of free radicals in biological macromolecules and their constituents.

Publications:

Riesz, P., Smitherman, T. C., and Scher, C. D.: Hydrogen transfer from exchangeable to carbon-bound sites in γ-irradiated proteins and nucleic acids: The mechanism of radical saturation. Int. J. Radiat. Biol. 17: 389-393, 1970.

Scher, C. D., and Riesz, P.: Hydrogen transfer from exchangeable to carbon-bound sites in γ-irradiated dry proteins. Radiat. Res. 170: 35-49, 1970.

Riesz, P., and White, F. H., Jr.: Radical distributions in γ-irradiated dry proteins at 195°K. Radiat. Res. 170: 24-34, 1970.

Holroyd, R., Glass, J., and Riesz, P.: Radicals formed in proteins by reaction with hydrogen atoms. Radiat. Res. 170: 59-67, 1970.

White, F. H., Jr., Kon, H., and Riesz, P.: Comparison of electrical discharge with γ-radiation for the production of free radicals in lyophilized proteins. Radiat. Res. 45: 8-24, 1971.

Smitherman, T. C., Moss, H. M., and Riesz, P.: Radiation-induced hydrogen transfer in nucleic acids, proteins and related substances: Effects of temperature and scavengers. Radiat. Res. (accepted for publication).

Miller, J. H., White, F. H., Jr., Riesz, P., and Kon, H.: Distributions of free radicals among amino acids from lyophilized ultraviolet-irradiated proteins. J. Photochem. Photobiol. (accepted for publication).

ANNUAL REPORT

THE ENDOCRINE EVALUATION BRANCH AND BREAST CANCER TASK FORCE PROGRAM
July 1, 1970 - June 30, 1971

INTRODUCTION

During the year under review the Breast Cancer Task Force has undergone a profound reorientation, based on a shift of priorities toward rapid improvement of the clinical management of breast cancer patients. A strong in-house program on Breast Cancer as well as increased activities in collaborating institutions is central to the new plans. The Endocrine Evaluation Branch has continued to carry out the Task Force Program with respect to contracts and collaborative activities.

PROGRAM AIMS AND RESOURCES

The general aims of the Breast Cancer Task Force—improved diagnosis, prognosis and treatment through clinical research and experimental biology—remain the same, but the shift in emphasis towards urgent improvement of clinical treatment has required drastic changes in organization. An internal "Steering Committee" consisting of ten Staff members has met weekly to analyze the Breast Cancer problem in detail, with the aim of summarizing the revised program in a new Research-Logic Framework Chart. Fresh informational input was sought in discussions with Task Force advisors and in seminars with specialists in fields as varied as thermography, radiation therapy, biochemical discriminants, endocrine ablation therapy, and model tumor systems. While the Steering Committee sought to sharpen the approach to clinical treatment the Task Force members were asked to develop a complete and basic biological program. They have outlined a program of studies related to the demonstration of "B" particles in human milk, to the histogenesis of human mammary cancer, to the physiological classifications of human mammary cancers, to the immunological study of human and experimental neoplasms, and to host-tumor interactions in mammary cancer. Before the end of the current fiscal year it is planned that the Task Force and Steering Committee, meeting together, will work out an integrated program with priorities set on objectives both in clinical medicine and in the general biology of breast cancer.

The Chief of the Endocrine Evaluation Branch is the Executive Secretary of the Breast Cancer Task Force, and he works with the Chairman to carry out the objectives of the Task Force. The Endocrine Evaluation Branch guides the development of contracts, working arrangements with clinical groups, liaison with other NCI groups, and within the limits of its resources acts as a clearing house for information relating to breast cancer. The Branch also carries out office functions connected with meetings of the Task Force and its committees, the Steering Committee and the Breast Cancer Task Force Contract Review Committee.

The Endocrine Evaluation Branch consists of the Office of the Chief and two sections. The Section on Endocrine-Related Tumors supports projects utilizing model breast tumor systems in studies of host-tumor biology, exploratory drug

therapy and especially preneoplastic lesions. The Section on Biological Activities of Hormones is concerned mainly with the measurement of factors such as intracellular hormone receptors or urinary steroids as diagnostic or prognostic aids. In the Office of the Chief there is a Coordinator for Cooperative Clinical Activities who acts as liaison with the Pathology Working Group (a Task Force committee) and attempts to bring special clinical viewpoints, such as that of epidemiology, to bear on the development of program.

CONTINUING RESEARCH DURING DEVELOPMENT OF THE NEW PROGRAM

During the over-all reconstruction of program a number of older projects are continuing while others are being phased out because of low priority in the new program. These projects fall generally into three categories: discriminants for clinical prognosis, tumor models for the study of the biology of breast cancer, and preclinical drug therapy.

Discriminants for Prognosis. The discriminant program aims at finding means of anticipating the course of disease at various stages and for different conditions of treatment. Discriminants that have been proposed include those based on a tumor factor (e.g., estrogen-receptor levels), and those based on host condition or host-cancer interaction, as shown by urinary steroids and patterns of clinical and histopathological data. Discriminants based on urinary steroids are often referred to as "Bulbrook discriminants" although the theory and methodology may differ greatly from those originally used and the steroids may constitute only one of the factors entering into a discriminant.

The attempt by Dr. Elwood Jensen of the University of Chicago to predict the response of a patient to adrenalectomy by assaying the estrogen-receptors of biopsied tumor, appears to be close to validation. Eight out of 10 patients with high receptor titer showed regression of disease after endocrine ablation, while only one of 27 with negative or very low readings had a favorable response. It appears likely that careful use of this test would make it possible to eliminate most of the surgical ablations for advanced breast cancer that would fail to help the patient.

Patients undergoing mastectomy for primary breast cancer may have a "free period" lasting a few months up to several years. If the physician knew his patient was in the high risk category at this time he would have good reason for instituting additional therapy at once instead of waiting for progression. The approach taken in the Primary Breast Cancer Study was to determine whether the high risk patient could be distinguished through the use of a discriminant based on urinary steroids and the tissue pathology of the tumor and lymph nodes. Clinicians of three institutions—Alton Ochsner Clinic, Yale University and the University of Pennsylvania—furnish the urine specimens and tissue samples from their patients in the study. The tissues are processed at the hospitals of origin and sent to the St. Vincent Hospital (Worcester) for confirmation and study, and the urine specimens are sent to the Worcester Foundation for Experimental Biology for steroid assay profiles. Documentation and statistical work for the project are carried out at Louisiana State University. The services for steroid analysis, histopathology and documentation are

supported by contract. Scheduling of services is kept in balance by the LSU Statistical Center and the Endocrine Evaluation Branch. A supplementary project has been started at Lackland Air Base to include women with non-cancerous lesions of the breast and other controls. Preliminary discriminants worked out by Dr. McMahan of LSU show promise of being able to indicate patients in the high risk category within the first year with 90% accuracy, and within two years with over 95% accuracy.

A second contract facility for automated steroid analysis was set up at the Research Foundation for Mental Hygiene in Orangeburg, N. Y. This has made it possible to extend the urinary discriminant approach to three other projects, all too new for even preliminary results. One of these was organized by the Section on Biological Activities to determine the validity of the Bulbrook discriminant, or another steroid-based discriminant, as a predictor of success for hypophysectomy or adrenalectomy in advanced breast cancer. The second, an additional control for the primary Breast Cancer Study, will study the urinary steroid pattern in endometrial cancer. The third project is based in Israel, where endocrine and anthropometric studies are being carried out on three ethnic groups—Yemenites, who have a low incidence of breast cancer, other Asians who have 3.5 times as much, and Israelis of East European extraction who have 6.5 times as much. Urinary specimens from these groups are sent to the Orangeburg facility for steroid analysis.

Finally, there is a need to make it possible to recognize when a regression is under way, since detectable response to treatment is not always rapid or dramatic. An attempt is being made at Jefferson Medical College to find an early indication of response—in this instance, to see whether changes in the emission of heat from a tumor can be used to indicate responsiveness to a drug, in a matter of days rather than weeks. The pattern of heat change in the area of a breast tumor sometimes does change when hormones are administered, but data are not yet sufficient to show whether or not these changes are related to regression of the tumor.

Tumor Models for Research and Testing. Model tumor systems are essential, for studies on the biology of breast cancer as well as for the testing of drugs. The Section on Endocrine-Related Tumors has investigated many neoplasms of mammary tissue in its contract projects and utilizes some of them regularly for research and testing. Since most of the useful endocrine-related tumor systems are found in inbred rats, a tumor bank and host-genetics monitoring facility was set up at the Mason Research Institute in Worcester. This supplements the facilities set up by CCNSC for monitoring mouse stocks and their tumors. The tumor bank contains specimens of 51 tumors, many of them unobtainable from other sources. The genetic quality of such inbred rat strains as the Fischer/344, Buffalo, and August 28807, from commercial breeders, is monitored by reciprocal skin grafting, blood typing and cytogenetic studies.

DMBA-induced mammary tumors in Sprague-Dawley rats serve as a model for primary breast cancer and consequently are much studied. Investigators at the Southern Research Institute are studying the cell kinetics of such tumors as affected by ablative surgery and drug therapy. A DMBA-induced tumor in the Fischer/344 rat, the 13762 mammary adenocarcinoma, has been used as a transplantable model system for a variety of purposes at the Mason Research

Institute; it has been used routinely at the Mason Research Institute for testing new compounds and is currently being used to study the effects of surgery, chemotherapy and immunotherapy on metastases that appear after removal of the primary graft. It was found this year that this tumor has an anti-uterotrophic effect, apparently by depriving the uterus of endogeneous estrogens—an effect for which there is also some evidence in patients with human breast cancer.

Another model system used at Mason Research Institute, the R-35 mammary adenocarcinoma of the Sprague-Dawley rat, was found to contain a "C-type" virus. This was one result of collaboration between the Section on Endocrine-Related Tumors and the Special Virus Cancer Program (Etiology) to screen all tumors available at EEB contract facilities for viruses and to find viruses in human breast tumors and milk. Three of the contract facilities used for these purposes by the Special Virus Cancer Program—at Georgetown University, Institute for Medical Research, and Charles Pfizer & Co.—have been supported in whole or in part by Breast Cancer Task Force funds. Many of the rat tumor systems utilized over the years in the Endocrine Evaluation Branch program and others at NCI originated in Dr. Dunning's laboratory at the University of Miami. This contract facility will be supported in the future by the Special Virus Cancer Program for an extensive study of viruses in induced and autochthonous rat tumors.

The Breast Cancer Task Force has a Pathology Working Group which has the problem of early breast cancer lesions under constant review. In the contract program two projects have been supported at the University of California, where the "hyperplastic alveolar nodules" of DeOme became known as the outstanding animal model of early lesions. These projects aim at utilizing new techniques to study sub-gross and microscopic lesions in the breasts of dogs and human beings, to establish norms of preneoplastic manifestations in these species. An "Atlas of Subgross Pathology of the Human Breast" is being prepared, based on studies of 295 specimens from accident victims or routine autopsies.

Preclinical Drug Therapy. Due to a policy decision, activities of the Branch having to do with procurement and screening of endocrine compounds for clinical use are being phased out. The contracts for synthesis of steroidal antitumor agents (Research Triangle Institute), large scale re-synthesis of steroid derivatives (Upjohn) and quality control of hormonal agents (University of Tennessee) are on a terminal year. During the past year only 5 new steroids and 1 non-steroid were received from contract sources, and there were 4 and 3 repeat orders of steroids and non-steroids received. Twenty non-steroids in small amounts were received from outside sources for screening in endocrine-related tumor systems, and a kilogram of a phthalimide derivative (thought to be an inhibitor of pituitary prolactin) was received from Chemie Grünenthal GMBH for tumor experiments. Only one new compound—estradiol mustard—was entered from this program into clinical trial during the past year; however two clinical cooperative groups are continuing studies with the cholesteryl mustard, phenesterin.

Immunotherapy. Since the last report a contract has been let with Hebrew University (Hadassah Medical School, Jerusalem) for "Pre-clinical Studies on

a Tumor-Protective Activity of a Methanol Insoluble Fraction of Attenuated Tubercle Bacilli (BCG)." The immediate objective is to develop appropriate animal models for the study of adjuvants to immunological protection against breast cancer. Dr. Weiss is using mice in this contract; Dr. Bogden at Mason Research Institute is carrying out studies with rats and a methanol-soluble fraction of M. butyricum and H. pertussis.

SUMMARY OF PROGRESS AND FORECAST OF DIRECTION

During the past year the basis has been laid for a complete reconstruction of the Breast Cancer Task Force Program, and the Endocrine Evaluation Branch has played a part in this. It is expected that this Branch will continue to manage research projects having to do with the biology of breast cancer, in directions required by the Task Force and in coordination with the new in-house clinical program on breast cancer. Discriminant projects aimed at prognosis regarding the patient's risk of recurrence in primary breast cancer, or ability to respond to drugs or ablative therapy, have been kept in full swing this year and will be essentially validated or rejected by the end of the next year. The Branch will continue to assist the Special Virus Cancer Program in its studies of animal and human sources of oncogenic viruses, and in endocrine factors involved in carcinogenesis. Preclinical activities of the Branch relating to procurement and screening of compounds of endocrine interest will be phased out, but breast tumor models will continue to be used for studies in host-tumor relationship and exploratory studies of drug action and therapeutic modalities.

Contract #64-46 - Hospital for Joint Diseases

1. Biswas, Shibabrata and Hollander, Vincent P. Purification and properties of a lymphoid acid ribonuclease. J. Biol. Chem. 244: 4185-4192, 1969.

2. Biswas, Shibabrata and Hollander, Vincent P. Studies on an acid ribonuclease from corticoid-sensitive 9α-Fluoroprednisolone-treated lymphosarcoma P1798: Changes in heat inactivation kinetics of the enzyme due to steroid treatment. Cancer Res. 30: 827-829, 1970.

Contract #68-1304 - Jefferson Medical College

1. Mansfield, Carl M., Wallace, John D., Curley, Robert F., Kramer, Simon, Southard, Martha E. and Driscoll, Dorothy. A comparison of the temperature curves recorded over normal and abnormal breasts. Radiol. 94: 697-698, 1970.

Contract #65-6 and #67-1146 - Mason Research Institute

1. Rosenkrantz, Harris. Physiology of prostatic phosphohydrolase secretion. Ann. N.Y. Acad. Sci. 166: 466-481, 1969.

2. Gouvelis, Alexander, Baker, John R. and Rosenkrantz, Harris. Histochemistry of the canine prostate. Invest. Urol. 8: 426-441, 1971.

3. Chopra, Harish C. and Mason, Marcus M. A new virus in a spontaneous mammary tumor of a Rhesus monkey. Cancer Res. 30: 2081-2086, 1970.

Contract #66-71 - Southern Research Institute

1. Simpson-Herren, Linda and Griswold, Daniel P., Jr. Studies of the kinetics of growth and regression of 7,12-Dimethylbenz(a)anthracene-induced mammary adenocarcinoma in Sprague-Dawley rats. Cancer Res. 30: 813-818, 1970.

2. Griswold, Daniel P., Jr. and Green, Charles H. Observations on the hormone sensitivity of 7,12-Dimethylbenz(a)anthracene-induced mammary tumors in the Sprague-Dawley rat. Cancer Res. 30: 819-826, 1970.

3. Simpson-Herren, Linda and Lloyd, Harris H. Kinetic parameters and growth curves for experimental tumor systems. Cancer Chemo. Reports 54: 143-174, 1970.

Contract #43-2395 and #65-1050 - E. R. Squibb and Sons

1. Hilf, Russell, Goldenberg, H., Bell, C., Michel, I., Orlando, R. A. and Archer, F. L. Some biochemical characteristics of rodent and human mammary carcinomas. Enzymologia biologica et clinica, 11: 162-176, 1970.

Contract #43-2395 and #65-1050 - E. R. Squibb and Sons (Continued)

2. Hilf, Russell, Goldenberg, Harold, Gruenstein, Margot, Meranze, David R. and Shimkin, Michael B. Lack of correlation between morphological and biochemical parameters in mammary adenocarcinomas of rats induced with 7,12-Dimethylbenz(a)anthracene. Cancer Res. 30: 1223-1230, 1970.

3. Hilf, Russell, Goldenberg, Harold, Michel, Inge, Orlando, Robert A. and Archer, Francis L. Enzymes, nucleic acids and lipids in human breast cancer and normal breast tissue. Cancer Res. 30: 1874-1882, 1970.

4. Hilf, Russell, Goldenberg, Harold, Michel, Inge, Gruenstein, Margot, Meranze, David R. and Shimkin, Michael B. Biochemical events associated with regression of 7,12-Dimethylbenz(a)anthracene-induced mammary carcinomas after ovariectomy. Cancer Res. 31: 52-58, 1971.

Contract #66-968 - Worcester Foundation for Experimental Biology

1. Roberts, Jeremy B. and Zydlewski, Walter R. Multiple wavelength spectrophotomethy as applied to chromatogram scanning. Am. J. Clin. Path. 53: Part 2, 1970.

In Collaboration with Etiology

Contract #67-1176 - Chas. Pfizer & Co., Inc.

1. Chopra, H. and Taylor, D. Jane. Virus particles in rat mammary tumors of varying origin. JNCI 44: 1141-1147, 1970.

Serial No. NCI-15-000
1. NCI
2. GL&C
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Part A

Project Title: Screening

Principal Investigator: Dr. Nathaniel I. Berlin

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

Serial No. NCI-15-001
1. NCI
2. GL&C
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Part A

Project Title: Screening

Principal Investigator: Dr. Nathaniel I. Berlin

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

Serial No. NCI-15-002
1. NCI
2. GL&C
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Part A

Project Title: Emergency Admissions

Principal Investigator: Dr. Nathaniel I. Berlin

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

SUMMARY STATEMENT
ANNUAL REPORT
Dermatology Branch - NCI
July 1, 1970 - June 30, 1971

The Dermatology Branch studies the growth and differentiation of epithelium and lymphoreticular tissues in normal hyperplastic and neoplastic states. The major thrust of the Branch effort is to study the skin from the point of view of its basic biology and its clinical diseases. In addition to its research efforts, the Branch serves as the dermatologic consultant to the Clinical Center. In the past several years two biochemists working within the Branch, Dr. Carl Levy, Dr. Simon Rothberg have left and have been replaced by Dr. William Levis, a dermatologist interested in immune mechanisms within the skin and Dr. Gary Peck, a dermatologist interested in tissue culture and chemistry of the skin. In addition Dr. Bruce Wetzel, an electron microscopist who has just joined the Branch is interested in the ultrastructure of skin and in the biology of the eosinophil, neutrophil and mast cell.

At present the major specific clinical and laboratory interests of the Dermatology Branch are (1) The Biochemistry of Normal and Diseased Skin: To extract, isolate and chemically characterize the keratohyalin granule; To study the chemistry of the collagen molecule; To study the influence of Vitamin A on keratinization; To study the incorporation of amino acid isotopes into biopolymers within the epidermis. (2) Elucidation of the Gene Defects in Inherited Diseases of the Skin (genodermatosis): To study the ultrastructure of the autosomal dominant skin disease, Darier's disease; To study malfunctions of the DNA-repair enzyme complex in Xeroderma Pigmentosa; To study the generation of cyclic AMP in urine of patients with the Basal Cell Nevus Syndrome; To investigate the lysosomal activity in Epidermolysis Bullosa Simplex; To study the synthesis of collagen in Osteogenesis Imperfecti. (3) The Immunologic Role of the Lymphocyte: To study the ultrastructure of cell interrelationships and cell contacts in human leukocyte cultures undergoing blastogenesis in response to phytohemagglutinin, antigens and allogeneic cells; To investigate the mechanism concerned with the control of mitosis, differentiation, function, and potentiality of the normal and pathologic cells with particular reference to the lymphocyte and cells of the lymphoreticular system; To investigate the functional and morphological interactions between lymphocytes and macrophages among different populations of lymphocytes during in vitro immune responses related to delayed hypersensitivity and homograft reaction. (4) The Ultrastructure of Normal and Diseases Skin: Study of the ontogeny of giant melanin granules in the Beige mouse, an animal model for the Chediak-Higashi disease; To study the ultrastructure of the blistering which takes place within the upper layers of the skin of the mouse following injection of a Group II staphlococcal exotoxin. This is an animal model for the study of Toxic Epidermal Necrolysis or Ritter's Disease, a disease of the skin of infants and children; To examine tissues from benign and malignant diseases of the skin; To identify the ubiquity or specificity of the so-called Sezary and MF cell; To study the ultrastructure of Toxic

Epidermal Necrolysis resulting from Graft-Versus-Host Reaction in the human; To study the ultrastructure of cells undergoing transformation through the mechanism of in vitro carcinogenesis produced by dimethylbenzanthracene and other solvents and to attempt to identify the epidermal or mesenchymal origin of these transforming cells. (5) The Development of Therapy for Specific Skin Diseases: To treat selected psoriatic patients with topical CCNU in order to evaluate the safety and effectiveness of the drug in psoriasis. To evaluate the use of topical nitrogen mustard in treating the lesions of psoriasis in selected patients; To test topical BCNU and CCNU in treatment of skin cancers. (6) Study of the Pathogenesis of Specific Skin Diseases: To review the natural history, pathologic features and response to therapy in a series of 144 Mycosis Fungoides patients treated at NIH.

1. Biochemistry of Normal and Diseased Skin: Keratin is the chief metabolic product of the epidermal cell or keratinocyte. Within the granular layer of the epidermis, epidermal cells contain large amounts of a peculiar body known as the keratohyalin granule (KHG). The chemistry and function of the KHG has never been elucidated completely. In our laboratory, we have developed a method for extracting and reaggregating keratohyalin granules in vitro. Further, it has been found that the aggregates are composed of an oligomeric series of protein containing thirteen members. This oligomer has been purified and found to have a unique amino acid composition, namely large amounts of serine, histidine, arginine and glycine. By the method of immunofluorescence we have been able to identify the isolated macroaggregates as being in fact identical to the in situ KHG. The function of the KHG remains unknown. In the past it has been considered to be important in forming the final keratin product in the epidermis. Since we have found that keratohyalin is composed of a basic protein complexed with RNA, an alternate hypothesis arises that the bound RNA plays a role in controlling epidermal cell growth and development and that the basic protein acts to specifically bind nucleotides.

In cooperation with NIDR the disease Osteogenesis Imperfecti, an autosomal dominant disease in the human is being studied. This disease is characterized by fragile bone tissue and blue sclera. When fibroblasts from patients with Osteogenesis Imperfecti are cultured, they show a defect in the amount of collagen synthesised. Also the effect of pH on the precursors of collagen biosynthesis in vitro and on the enzymes of collagen biosynthesis is being examined.

2. Elucidation of the Gene Defect in Inherited Diseases of the Skin (genodermatosis): Darier's Disease, an autosomal dominant disease of the skin has been studied with the electron microscope. In this disease a split takes place in the suprabasalar area of the epidermis. Peculiar acantholytic cells known as corps ronds and grains are formed. Previously it was suspected that these peculiar cells form as a step wise result of a defect in the desmosome-tonofilament complex. Our data, however, indicates that the earliest findings are vacuoles within basal cells. In some instances these vacuoles enlarge filling the entire cell. These large vacuolated cells correspond to the corps ronds of light microscopy. Some of the vacuolated cells do not convert to corps ronds but instead the

vacuoles apparently collapse, and some keratinization takes place. These dyskeratotic cells are known as grains. It appears that both grains and corps ronds originate from vacuolated keratinocytes. The gene defect in this disease may be related to the formation of abnormal cytoplasmic vacuoles whose chemical nature remains at present unknown.

Lysosome activity in Epidermolysis Bullosa Simplex, a genetic disease of the skin has been studied using the electron microscope. Acid phosphatase, a marker enzyme for lysosomes has been studied in this disease and found to be normal. An unexpected finding occured in the study; i.e., the observation that there is apparently a nonspecific attraction of the melanin granule for lead nitrate used in the standard histochemical test for acid phosphatase. This observation opens questions concerning findings by others using this method in studying acid phosphatase and melanin granules.

Osteogenesis Imperfecti is being studied as to its collagen chemistry as discussed above.

Xeroderma Pigmentosa an autosomal recessive disease of the skin involving ultraviolet-induced skin cancers will be discussed in the section below.

The generation of cyclic AMP in the urine of patients of Basal Cell Nevus Syndrome has been found to be normal.

3. Immunologic Role of the Lymphocyte: Peripheral lymphocytes have been used for the first time to test the ability of XDP patients to repair UV-induced damage of DNA. We have found what is apparently the same defect as that described in skin cells, to be present in lymphocytes in three of four patients with XDP. In addition, we have discovered a new form of XDP, evidenced by our fourth patient. While this patient's clinical symptoms are as least as severe as those of the other three, the lymphocytes appear to have the same rate and duration of DNA repair. This indicates that XDP may be a genetically heterogeneous disease.

A new millipore filter assay technique has been developed in our laboratory and is now being widely used by many other laboratories in the field.

The ultrastructure and extent of intercellular contacts between the lymphocytes and macrophages have been analyzed by serial electron photomicrographs of cultured blood cells. Complex loculations and appositional structures have been found.

4. The Ultrastructure of Normal and Diseased Skin: The electron microscope was used to study the effects of mutant beige gene on melanin granule formation in the eye of the mouse. The Beige mouse is a model for the Chediak-Higashi disease of man. In addition a number of other melanin mutants such as the Ruby, the Pink and the Albino were also studied as to the structures of their abnormal melanin granules. Also the combined effects of the beige gene with each of these other three genes were studied. It has been found that animals mutant for both the beige and ruby gene have

giant granules of the ruby type; whereas animals mutant for both the beige and albino, and beige and pink genes do not have giant granules. Instead there is an apparent abnormality in fibril formation which proceeds melanization.

The electron microscope has been used to examine the Mouse model developed by Dr. Melish, for the study of Ritter's Disease, an exfolative skin disease of children. It has been found that this disease is caused by a Group II staphlococcal exotoxin. In newborn mice this exotoxin has been shown to produce exfoliation of the skin of these mice similiar to that which occurs in children. We have been studying with the electron microscope the early effects of this exotoxin which apparently causes separation, exclusively, of cells within the granular layer. Five minutes following injection there appears a peculiar segmentation of one desmosomal line, followed by splitting of the desmosome into halves and separation of the cells from one another.

We have continued to study skin from patients with Mycosis Fungoides, Sezary Syndrome, Parapsoriasis of various types and control skin to see if the so-called MF or Sezary cell is a ubiquitous cell or rather specific for Mycosis Fungoides. Although the cell is found in large numbers in Sezary Syndrome, Mycosis Fungoides and Parapsoriasis En Plaque tissues, it has also been found in increasing numbers in benign diseases of the skin. So it appears that the cell is not specific for MF and its diagnostic potential seems to be doubtful.

The first reported case of Toxic Epidermal Necrolysis developing in a patient with Graft-Versus-Host Disease following bone marrow transplantations has been studied with the electron microscope.

Mouse epidermal cells were transformed in vitro by the carcinogen dimethylbenzanthracene. These transformed cells were then injected subcutaneously into isologous mice. Resulting tumors were biopsied and resuspended in culture. At all stages these cells and tissues were monitored by electron microscopy in an attempt to establish the nature and origin of the cells undergoing malignant transformation. By identifying tonofibrils and desmosomes, the transformed cells have been identified as being epidermoid in nature.

The effect of vitamin A acid on the induction of mucous metaplasia in 13-day chick metatarsal skin was studied in organ culture using the electron microscope. It was found that whereas control specimens keratinize normally with the formation of thick bundles of tonofilaments, keratohyalin granules and a multi-layered stratum corneum, vitamin A acid treated specimens did not undergo keratinization. Stratum corneum and keratohyalin granules were not formed, and mucous droplets were present in the cytoplasm.

5. Therapy for Specific Skin Diseases: A group of selected psoriatic patients were treated with topical nitrogen mustard in a double blind study. It was found that nitrogen mustard was effective in reversing the psoriatic lesions. However, since most of the patients developed delayed hypersensi-

tivity, the use of this drug does not seem practical.

Some success was found in treating basal cell carcinomas with BCNU and CCNU. However, since the stability of these chemicals in the vehicle was impossible to control the study was discontinued.

6. Specific Skin Diseases: In a review of 144 patients with Mycosis Fungoides seen at NIH, it was found that the survival of patients following diagnosis was approximately the same during the years 1956 to 1962 as during the years 1963 to 1968. Correlating with this is a finding that long term survival is not dramatically different whether the initial treatments is with electron beam, cytoxan or methotrexate.

Serial No. NCI-3607
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tissue Culture Studies of Human Lymphocytes

Previous Serial Number: Same

Principal Investigator: Jay H. Robbins, M.D. **Co-Investigator:** William R. Levis, M.D.

Other Investigators: Peter G. Burk, M.D., and Peter M. Elias, M.D.

Cooperating Units: Stuart H. Yuspa, M.D., Biology Branch, NCI; Rosemary Cuddy, Program Analysis and Reporting Section, EA, NCI.

Man Years (July 1, 1970 through June 30, 1971)

Total:	3.6
Professional:	2.9
Other:	.7

Project Description:

Objectives:

1) To investigate the mechanisms concerned with the control of mitosis, differentiation, function, and potentialities of normal and pathologic cells, with particular reference to the lymphocyte and cells of the lymphoreticular system.

2) To investigate the functional and morphological interactions between lymphocytes and macrophages and among different populations of lymphocytes during in vitro immune responses related to delayed hypersensitivity and to homograft reactions.

3) To study the DNA excisional repair process in normal lymphocytes and in lymphocytes from patients with xeroderma pigmentosum (XDP); to correlate the findings of these studies on lymphocytes with the findings obtained in skin fibroblasts and epidermal cells.

Material:

Lymphocytes and other cells are obtained from hospitalized patients and outpatients.

Methods Employed:

Human peripheral blood lymphocytes are cultured in vitro and stimulated to transform into large blastoid cells by factors such as phytohemagglutinin, antigens, and foreign tissue. This phenomenon of blastogenesis is evaluated by the morphologic and biochemical changes which the lymphocytes undergo. The mechanisms by which these blastogenic factors act and the culture conditions required for blastogenesis are determined.

Human peripheral blood leukocytes are separated into a glass-adherent cell population, composed largely of macrophages, and into a population consisting of small lymphocytes. By recombining such populations obtained from the same and from different individuals, the function of each population is determined. Interactions and contacts between cells are examined by light and electron microscopic techniques, including methods involving the in situ fixation and embedding of the cultured cells in order to preserve the intercellular contacts and spatial relationships of the cells.

Normal and XDP patients' cells are irradiated with ultraviolet (UV) light and then incubated with radioactive thymidine. The degree of incorporation of radioactive label into the DNA of the cells is determined by autoradiography and by scintillation counting. The effect of UV-radiation on the ability of normal and XDP patients' lymphocytes to undergo semiconservative DNA synthesis is studied using blastogenic factors such as phytohemagglutinin to induce semiconservative synthesis.

Major Findings:

1) Peripheral blood lymphocytes have been used for the first time to test the ability of XDP patients to repair UV-induced damage to DNA. A defect in such repair, which may be causally related to the development of the multiple cutaneous malignancies characteristic of this disease, had heretofore been studied only in cells from skin. We have found what is apparently the same defect as that described in skin cells to be present in lymphocytes from three of four patients with XDP. The suitability of peripheral blood lymphocytes for studying this defect in XDP has made it possible to utilize a very rapid and sensitive scintillation assay procedure for measuring ultraviolet-induced thymidine incorporation, a parameter related to the replicative step in the repair of UV-damaged DNA. Using this procedure, we have found that XDP lymphocytes which have an abnormally low rate of repair of UV damage continue their repair for a longer time than normal lymphocytes, so that the total amount of thymidine eventually incorporated into the XDP cells seems to be comparable to that incorporated into normal lymphocytes.

2) We have discovered a new form of XDP evidenced by our fourth patient. While his clinical symptoms were at least as severe as those of the other three patients, his lymphocytes appeared to have the same rate and duration of DNA repair as normal lymphocytes. In order to determine whether this unexpected finding was unique to the lymphocytes, autoradiographic studies of the UV-induced tritiated thymidine uptake in this

patient's skin fibroblasts were undertaken. These studies indicated that these skin fibroblasts also had a normal rate of UV-induced thymidine incorporation. Thus, this patient may have a different defect in the repair process from the other patients, and XDP may be a genetically heterogeneous disease.

3) The millipore filter assay technique, first utilized and applied in this laboratory to measure the incorporation of tritiated thymidine into DNA of lymphocytes undergoing semiconservative DNA synthesis, has been modified to make it more rapid and sensitive. By combusting the filter and its contained radioactive DNA, the tritium in the DNA is incorporated into water and can then be measured by scintillation spectrometry. This combustion method results in several advantages in the counting of the radioactive sample, the most important being a two- to three-fold increase in absolute counting efficiency over that previously obtained. A second improvement of the original assay technique is the substitution of precision crafted metal funnels for the porcelain Buchner funnels. The dimensions of each metal funnel are such that a millipore filter, when simply dropped into the funnel without any time-consuming placement on the part of the operator, must of necessity cover each and every hole of the supporting screen, thereby making it impossible for any of the applied sample or washes to by-pass the filter. A third improvement presently being developed is the automation of the wash procedures which should make it possible for the number of samples which can be assayed by a single individual to be increased from the present 24 samples per hour to at least 64 per hour.

4) The ultrastructure and extent of the intercellular contacts between lymphocytes and macrophages has been analyzed from multiple and serial electron photomicrographs of cultured human peripheral blood leukocytes fixed and embedded in situ on glass coverslips. Complex appositional zones, consisting of point and area contacts and involving large areas of the lateral surfaces of juxtaposed cells, were found to contain numerous anastomosing intercellular channels and lakes. The relative isolation of such intercellular loculations from the surrounding large pool of culture fluid contains and prevents the dilution of high concentrations of biologically active substances known to be released by cells in these cultures. In these loculations, therefore, substances such as lymphotoxin, migration inhibitory factor, transfer factor, etc., may be able to reach and affect the target cells in the required, effectively high concentration.

Significance to Cancer Research:

1) UV-radiation can induce malignancies in mammalian cells. In particular, patients with XDP develop multiple cutaneous malignancies on sun-exposed areas of skin presumably because of the effect of the UV-radiation in sunlight. Normal human cells have an excisional repair process which rapidly repairs certain types of UV-induced DNA damage while most patients with XDP have a marked impairment in the rate of such repair. Our studies on this and other possible repair processes in XDP and normal

cells may elucidate the relationship between the effectiveness of DNA repair and the development of malignancies, as well as the mechanism by which UV-radiation induces malignancies.

2) The lymphocyte is a cell involved in immune reactions which may be operative in the immune response to neoplastic tissue. Our studies concerned with blastogenesis and the control of cell division and differentiation of the lymphocyte may elucidate this cell's normal and abnormal functions in the immune response involved in the host's defense against cancer. More specifically the methodology we have developed may facilitate the elucidation of the mechanism of immunologic recognition including the manner in which a host normally recognizes a tumor as foreign.

3) Certain in vitro diagnostic and investigative studies concerning the response of lymphocytes to tumor antigens involve the measurement of DNA synthesis in lymphocytes. Furthermore, tissue typing to determine the most suitable donor for tissue and organ transplants including bone marrow transplants for leukemic patients often requires similar measurements. Our very rapid and sensitive assay technique for obtaining a measure of this DNA synthesis should therefore be of useful significance to cancer research in this regard.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Honors and Awards: None

Publications:

1. Burk, P. G., Lutzner, M. A., Clarke, D. D., and Robbins, J. H.: Ultraviolet-stimulated thymidine incorporation in xeroderma pigmentosum lymphocytes. J. Lab. Clin. Med., In Press.

2. Burk, P. G., Yuspa, S. H., Lutzner, M. A., and Robbins, J. H.: Xeroderma pigmentosum and D.N.A. repair. Lancet i, 600, 1971.

3. Robbins, J. H., Burk, P. G., and Levis, W. R.: Rapid and sensitive assay for D.N.A. synthesis in leukocyte cultures. Lancet ii, 98, 1970.

4. Robbins, J. H., and Levis, W. R.: "Delayed" peaks of DNA synthesis in phytohemagglutinin-stimulated human leucocyte cultures. Int. Arch. Allergy 39, 580, 1970.

5. Elias, P. M., Ornstein, L., Lutzner, M. A. and Robbins, J. H.: A method for studying the ultrastructure of intercellular contacts in leukocyte cultures. Experientia 27, 116, 1971.

Serial No. NCI-3612
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mycosis Fungoides: Prognostic Features, Autopsy Findings, and Response to Therapy

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Joseph D. Croft, Jr., M.D., Ervin H. Epstein, Jr., M.D. and David Levis, M.D.

Cooperating Units: Biometry Branch, NCI

Man Years (July 1, 1970 through June 30, 1971)

Total:	.01
Professional:	.01
Others:	

Project Description:

Objectives:

To review our experience with patients with mycosis fungoides.

Methods Employed:

Charts of all 144 patients with mycosis fungoides seen at the NIH have been carefully reviewed and pertinent information has been extracted. The latter has been transferred via punch cards to computer tape. With a punch card sorter and computer, appropriate correlations and summaries are being made.

Major Findings:

This group comprises by far the largest series of patients with mycosis fungoides that has been studied. Data so far accumulated indicates that survival could not be predicted by the patients' sex or race but that some other factors including age at diagnosis, duration of lesions before diagnosis, and type of lesions at time of diagnosis all are useful prognostic features. Survival of patients after diagnosis was approximately the same during the years 1956 to 1962 as during the years 1963 to 1968. Correlating with this is the finding that long-term survival is not dramatically different whether initial treatment is with electron beam, cytoxan,

or methotrexate.

Significance to Cancer Research:

By careful review of these patients, it is hoped that improved ability to predict outcome in the individual patients will result.

Proposed Course of Project:

The work is completed. A publication is being prepared.

Honors and Awards: None

Publications: None

Serial No. NCI-3613
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Extraction, Isolation and Characterization of Keratohyalin Granules.

Previous Serial Number: Same

Principal Investigator: Arthur Ugel, M.D.

Other Investigators: Drs. Rodbard and Chrambach.

Cooperating Units: Endocrinology Branch, NCI.

Man Years (July 1, 1970 through June 30, 1971)

Total:	3.74
Professional:	2.14
Others:	1.6

Project Description:

Objectives:

1) To verify the isolation of keratohyalin (KH) by immunological methods.

2) To characterize the physical-chemical properties of bovine KH

3) To compare the histochemical, ultrastructural and physical-chemical properties of bovine KH when isolated by various salt solutions

4) To further demonstrate homology of bovine KH by reversibility studies and amino acid analyses of the various bovine KH oligomeric states

5) To demonstrate homology of bovine KH oligomers by immunological methods

6) To develop an *in vitro* system for synthesizing labeled bovine KH in order to further study the cellular site of synthesis of KH, and to determine rates of synthesis of bovine KH; To study the incorporation of tritiated amino acids into in situ KH granules in order to better characterize the rate of synthesis and the composition of the in situ KH granule.

Methods Employed:

1) Crude bovine KH is isolated from cattle hoof epidermis by extraction with 1.0 M potassium phosphate buffer (pH 7.0). This extract is dialyzed against 32 volumes of distilled water which results in the in vitro aggregation of bovine KH. The resultant macroaggregates are injected into rabbits with Freud's adjuvant, and localization of antibody is performed by the indirect method on sections of cattle hoof epidermis and isolated macroaggregates of bovine KH.

2) Crude bovine KH is isolated by in vitro aggregation as described above. The macroaggregates are solubilized in sodium decylsulfate, then subjected to polyacrylamide gel electrophoresis at various gel concentrations.

3) Crude bovine KH is isolated as described above, except that the salts used for extraction are varied. In all cases, the initial extraction salt concentration is maintained at 1.0M. The various aggregates are studied by histochemical,ultrastructural, and physical-chemical methods as described.

4) Sodium decylsulfate solubilized bovine KH is fractionated on 9% polyacrylamide gels and the various species are rapidly precipitated by immersion in 7.5% acetic acid. The species are then sliced from gels, and extracted with sodium decylsulfate. These isolated species are then subjected to polyacrylamide gel electrophoresis as described above. Other isolated species (confirmed by polyacrylamide gel electrophoresis) are precipitated with 5% trichloracetic acid, washed, and analyzed for amino acids.

5) The various species of bovine KH are fractionated and isolated as described in 4. These species are then injected into rabbits, and indirect immunofluorescence is performed as described in 1.

6) Cattle hoof epidermis is minced, then placed in Media 199, or in similar media depleted of certain amino acids. Tritiated amino acids are then added, and incorporation is continued for various time periods. Samples of epidermis are then prepared for radioautographical studies, and other samples of epidermis are extracted with 1.0 M potassium phosphate buffer (pH 7.0) as described above (1).

Major Findings:

1) Immunization of rabbits with bovine macroaggregates results in the production of antibody directed against the in situ KH granule, as well as the isolated macroaggregate. The titer can be completely abolished by absorption with the isolated macroaggregate, and by no other material investigated to date. Fluorescence is limited to the in situ KH granule, and no other areas are positive. Cross-reactivity of bovine and rabbit KH can be demonstrated by indirect immunofluorescence.

2) Polyacryamide gel electrophoresis of bovine KH results in the fractionation of a 13 member homologous series of oligomers. Inspection of the fractionated species indicates that each size oligomer migrates with a small amount of conformational isomer. There is a linear relationship between the retardation coeffient (the slope of log mobility versus gel concentration), and oligomer number. This relationship has been used to determine the size of all the species of KH. These studies indicate that the monomer (subunit) has a molecular weight of 16,900. The extrapolated free mobility of all the species is constant. Crude bovine KH when fractionated on 9% polyacrylamide gels, also shows the presence of two nonhomologous species which appear to be conformational isomers of the samller oligomers. By varying the gel concentration and selectively unstacking these two nonhomologous species in 3% polyacrylamide gels, it has been possible to purify and isolate the entire group of 13 species in one step. The theoretical model developed to fit these data indicates that bovine KH should exist as a linear array of random coils in the presence of anionic detergent.

3) Regardless of the salt used to extract bovine KH, in vitro aggregation results in the formation of macroaggregates with histochemical characteristics of the in situ KH granule. At the ultrastructural level, those aggregates formed from 1.0M potassium phosphate extracts are the most ordered and homogeneous. Polyacrylamide gel electrophoresis of the various aggregates (solubilized in sodium decylsulfate) indicates that the KH obtained from potassium phosphate buffer is composed only of the homologous series of oligomers.

4) Isolation of high molecular weight oligomers of bovine KH, and refractionation results in the demonstration of the original high molecular weight oligomers, but small amounts of the lower molecular weight species are also present, and moderate amounts of the bovine KH monomer are seen. At no time can polymerization into higher molecular species be demonstrated. Amino acid analyses (corrected for loses of serine and tyrosine) and for tryptophan are identical for all the various species of bovine KH. The molecular weight of the subunit (monomer) of KH from these studies is 14,300. The analyses indicates a unique composition in the large amounts of serine, histidine, arginine and glycine that are present.

5) Isolation of the various species of bovine KH (as described above) and immunization of rabbits with these species results in the production of antibody to the entire in situ KH granule and the isolated macroaggregate.

6) Incorporation of tritiated histidine into bovine KH demonstrates label as early as 5 minutes, as demonstrated by isolation of labeled KH and autoradiographical methods. Incorporation continues at a linear rate for at least 24 hours. At no time is there labeling of the nucleus.

Significance to Cancer Research:

Presently, the function of KH is completely unknown. However, there are certain reasons for believing that KH may have a role as a "control" substance, based on both clinical and biochemical observations. Clinically, diseases of the skin which are characterized by atypical mitotic rates and fast turnover times (basal cell and epidermal cell cancer and psoriasis) are remarkable for the absence of morphologically recognizable KH they demonstrate at the histopathological level. A hypergranular skin, on the other hand, is usually one with a slow turnover rate. In resolving psoriasis, return of KH is noted. Biochemically, KH is composed of a basic protein complexed to RNA. It is entirely possible that the bound RNA plays a role in controlling the epidermal cell, and that the basic protein acts to specifically bind acidic nucleotides, possible turning off certain genes.

Proposed Course of Project:

Research will be continued along these lines.

Honors and Awards: None

Publications:

1. Ugel, A.R.: Keratohyalin: Extraction and *in vitro* aggregation. *Science* 166: 250, 1969.

2. Ugel, A.R., and Idler, W.W.: Stratum granulosum: Dissection from cattle hoof epidermis. *J. Invest. Dermatol.* 55: 350-353, 1970.

3. Epstein, E. Jr., and Ugel, A.R.: The effects of Mechroethamine on the lesions of psoriasis. *Arch. Derm.* 102: 504-506, 1970.

4. Ugel, A.R.: Isolation and characterization of oligoribonucleoproteins with histochemical and ultrastructural characteristics of keratohyalin. *J. Cell Biol.* In press.

5. Ugel, A.R., Chrambach, A., and Rodbard, D.: Characterization of an Oligomeric Series of bovine keratohyalin by polyacrylamide gel electrophoresis. *Anal. Bioch.* In press.

Serial No. NCI-3614
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Psoriasis Vulgaris: Treatment with Topical Nitrogen Mustard.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Arthur R. Ugel, M.D., and Ervin H. Epstein, Jr., M.D.

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:	.01
Professional:	.01
Other:	

Project Description:

Objectives:

To evaluate the efficacy of topically applied nitrogen mustard on the cutaneous lesions of psoriasis vulgaris.

Methods Employed:

A group of patients with typical psoriasis vulgaris were treated with topical applications of nitrogen mustard. A double-blind technique was employed.

Major Findings:

Topical applications of aqueous solutions of mechlorethamine hydrochloride were given to 12 patients with resistant psoriasis. Ten had definite improvement of their lesions, some with dramatic resolution. No systemic toxicity was detected in any patient but the development of hypersensitivity to this drug precluded its prolonged use.

Significance to Cancer Research:

This study may help understand the control of hyperproliferative states by the action of mechlorethamine on psoriasis.

Proposed Course of Project:

Due to the development of sensitivity to mechorethamine, the project has been terminated.

Honors and Awards: None

Publications:

1. Epstein, E. H., Jr. and Ugel, A. R.: Effects of Topical Mechlorethamine on Skin Lesions of Psoriasis. Arch. Derm., 102: 504, 1970.

Serial No. NCI-3617
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) Electron Microscopic Study of the Ultrastructure of Darier's Disease. 2) Study of the Development of Giant Melanin Granules in the Beige Mouse and the Study of a 'Double Mutant' Effect on Melanin Granule Formation in the Beige-Ruby, Beige-Pink and Beige-Albino Mice. 3) Continuation of the Study of Sezary Cells in Skin Lesions. 4) Study of the Ultrastructural Defect Caused by a Specific Exotoxin Formed by Group II Staphlococci in the Skin of Newborn Mice, a Model for Ritter's Disease of Infants.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Vincent Hearing, Ph. D., James Hobbs, M.D., Peter Horvath, M.D., Marian Melish, M.D., and Dr. Lillibridge.

Cooperating Units: Dermatology Department, Howard University Medical School. Division of Dermatology, Georgetown University, School of Medicine. Department of Pediatrics, University of Rochester, Medical School, Rochester, New York.

Man Years (July 1, 1970 through June 30, 1971)

Total:	4.2
Professional:	1.0
Other:	3.2

Project Description:

Objectives:

1) To study the ultrastructure of skin in the autosomal dominant genodermatosis, Darier's Disease, in an attempt to determine the derivation of the corps ronds and the grain with the intention of obtaining clues as to the possible chemical defect in this puzzling disease.

2) To study the effects of mutant pigment genes on the formation of melanin granules in the mouse eye, to obtain information as to how normal granules are constructed.

3) To examine skin in newborn mice injected with exotoxin derived from Group II staphlococci in order to determine how this toxin induces separation of individual cells of the granular layer from one another. Also to study children with Ritter's disease for which the newborn mouse serves as a model.

4) To continue the study of the relative number of Sezary cells present in tissues of patients with parapsoriasis of various types and other diseases of the skin in an effort to determine the specificity of this cell type for the lymphoma, mycosis fungoides.

Methods Employed:

1) Patients are sought with Darier's Disease, punch biopsies are taken and tissue is processed for electron microscopy.

2) Mice are especially bred for pigment mutations either 'single' or 'double' mutants. Eyes from these mice are processed for electron microscopy. Eyes from mutant mouse embryos are also studied.

3) Newborn mice are injected with exotoxin in Dr. Melish's laboratory. Biopsies are taken from skin and other organs of these mice at varying time periods following injection. Tissue is then processed for electron microscopy.

4) Biopsies are obtained from humans with MF, Sezary Syndrome, parapsoriasis and other diseases. This tissue is processed for electron microscopy.

Major Findings:

1) 19 lesions from 5 patients of 3 families with Darier's Disease were biopsied and studied by light and electron microscopy. Large cytoplasmic vacuoles were seen in basal cells. these vacuoles accumulated in increasing numbers in prickle and granular cells, which became acantholytic. In the granular layer, some cells were expanded by vacuoles which compressed existing tonofilaments and other cytoplasmic into a peripheral ring. These heavily vacuolated cells correspond to corps ronds. Other vacuolated cells continue to keratinize as evidence by formation of keratohyalin granules and aggregated bundles of tonofibrils which finally compressed the vacuoles into slits. These cells become ovoid, retained their nuclei and correspond to grains. Thus the present study indicates that grains and corps ronds can both originate from vacuolated keratinocytes. The gene defect in Darier's Disease may be closely related to the formation of this abnormal cytoplasmic vacuole.

2) Giant melanin granules form in the retina, iris, choroid and skin of the Beige mouse. Animals mutant for two pigment genes have been studied. Beige-ruby animals have giant granules of the Ruby type. However, in the beige-albino and beige-pink animals the granules are not giant in size.

In the pink and ruby mutants it has been determined that there is an abnormality of fibril formation proceeding melanization. This is currently being studied further.

3) It has been found in skin of the newborn mice injected with exotoxin from Group II staphlococcus that there is an unusual segmentation of one desmosomal lines. This occurs within 5 minutes of injection. The desmosome is then separated at the interdesmosomal line, half a desmosome remaining on each plasma membrane. The specificity of the desmosome as a target for the exotoxin is surprising, as is the restriction of the effect to granular cell desmosomes.

4) The Sezary cell has been found to be present in most all cases of parapsoriasis en plaque. Tissues from cases of Lichen Planus have also been found to have these cells, as well as one case of Porokeratosis of Mibelli, and one of reticulum cell sarcoma. It appears presently that the cell is not absolutely specific for the diagnosis of mycosis fungoides. Quantitation is being carried out.

Significance to Cancer Research:

Further study of genetic mechanisms in human genodermatosis should enable one to determine how normal keratinocytes operate and thus may eventually give clues as to the development of squamous cell and basal cell carcinoma. The study of the gene influence on melanin granule formation should provide insight as to the polygene effects on the formation of melanin granules and eventually might be able to be used in determining the role of melanin granule abnormalities in malignant melanomas.

Morphology of the desmosomes as dissected by the exotoxin enable one to study cell contacts which is apparently important in transformation of cells.

Diagnosis of diseases prodromal to MF would be important in evaluating early treatment of this lymphoma.

Proposed Course of Project:

The Darier's Disease project may continue if Dr. Gottlieb, one of the collaborating physicians is awarded a postdoctoral fellowship to work in our laboratories: otherwise, it will be discontinued. The melanin work will be continued if Doctor Hearing is able to obtain a postdoctoral extramural fellowship, otherwise this will be temporarily discontinued. Work on exotoxin and its effect on desmosomes should be completed this year. We will continue to look at new skin biopsies from various skin diseases and lymphomas in order to determine whether or not the Sezary Cell can be quantitatively used to predict MF lymphoma.

Honors and Awards:

None

Publications:

Lutzner, M. A., J. W. Hobbs,, and Horvath, P.: Ultrastructural characteristics of abnormal cells in sezary syndrome, mycosis fungoides, and parapsoriasis en plaque. Arch. Derm. 103: 375, 1971.

Serial No.NCI-3618
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Generation of Cyclic-AMP in the Urine of Patients with Basal Cell Nevus Syndrome (BCNS)

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Thomas Nigra, M.D., Ervin Epstein, M.D., Robert Marcus, M.D., Gerald Aurbach, M.D., and Richard N. Winickoff, M.D.

Cooperating Units: NIMD

Man Years (July 1, 1970 through June 30, 1971)

Total:	.02
Professional:	.02
Others:	

Project Description:

Objectives:

To determine if patients with BCNS are hyporesponsive to PTH extract as previously reported by Block and Clendenning in NEJM: 268, 1157-1162, 1963.

Methods Employed:

The recent report by Dr. Aurbach that patients with pseudohypoparathyroidism were hyporesponsive to parathyroid extract infusion with regards to generation of cyclic AMP in urine has lead us to evaluate the same group of patients with BCNS used by Block and Clendenning to determine if they too are hyporesponsive to PTH.

Twelve patients with BCNS will be admitted to the clinical center where routine medical evaluation will be done. In addition they will have skeletal survey, EEG, etc.

A PTH infusion and control day will result in collection of urine that will be assayed by Dr. Aurbach's laboratory.

Major Findings:

Patients with Basal Cell Nevus Syndrome are normoresponsive with regards to the excretion of Cyclic-AMP in urine to PTH.

Significance to Cancer Research:

Patients have multiple basal cell carcinomas. Any information relating to a possible chemical defect (gene-induced) in these patients might lead to new insight to mechanisms of tumorigenesis.

Proposed Course of Project:

Completed

Honors and Awards: None

Publication:

Aurbach, G. D., Marcus, R., Winicoff, R. N., Epstein, E. H. Jr., Nigra, T. P.: Urinary Excretion of 3' - 5' AMP in Syndromes Considered Refractive with the Parathyroid Hormone. Metabolism, 19: 799-808, 1970.

Serial No. NCI-3622
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Viral Etiology of Pemphigus

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Thomas Nigra, M.D., J. Sever, M.D., L. Barbosa, M.D., and N. Furcillo, M.D.

Cooperating Units: NINDS

Man Years (July 1, 1970 through June 30, 1971)

Total: .02
Professional: .02
Others:

Project Description:

Objectives:

Attempt to recover virus and conduct serological tests on patients with pemphigus.

Methods Employed:

In each case whole blood (10 ml) would be obtained, the serum separated and stored frozen for serologic surveys. Each specimen would be identified with the patient's name, the date of the collection and the study number. (Heparinized blood - 30 ml). The buffy coat is to be used for virus isolation and electron microscopy; the serum is to be used for virus isolation. Fluid from the blisters drawn and placed in SPAM, all of the material to be cultured in KB Hep-II and monkey kidney cultures with and without co-cultivation as identified by Siegl and Hahn. The sera will be tested for antibody to flu A, B and C, para I, II, III and IV and RS by the complement fixation method and to rubella and rubeola using the HI method.

Major Findings:

On three patients with pemphigus no evidence of viral etiology from above methods of screening have been found.

Significance to Cancer Research:

Possible relationship of parallelism to the viral etiology of cancer.

Proposed Course of Project:

Ongoing

Honors and Awards: None

Publications: None

Serial No. NCI-3623
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of Various Elastic and Collagen Tissue Diseases

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Thomas Nigra, M.D., Michael Friedland, M.D., Sam Narayan, M.D., and George Martin, M.D.

Cooperating Units: National Institute of Dental Research, Connective Tissue Section, Biochemistry Branch.

Man Years (July 1, 1970 through June 30, 1971)

Total: .96
Professional: .96
Others:

Project Description:

Objectives:

Purpose of the project is to determine steps involved in the formation of normal connective tissue as a baseline for study of disease states. Particular emphasis is placed on the chemistry of collagen and elastin, the enzymatic steps of cross linking, and the degradative processes.

Methods Employed:

The usual laboratory techniques of collagen chemistry are employed. Ostogenesis Imperfecta is an autosomal dominant disease of humans. It is characterized by fragile osseous tissue and blue sclerae. When diploid skin fibroblasts from patients with osteogenesis imperfecta are placed in tissue culture, there appears to be a defect in collagen biosynthesis. In comparison with normal collagen synthesis osteogenesis imperfecta fibroblasts produced approximately one-half as much.

Major Findings:

Collagenase is the major degradative enzyme found to date for the collagen molecule. This enzyme has been found in a number of tissues. Heretofore, no one has identified this particular degradative enzyme in human diploid skin fibroblast in culture. But we have isolated this

from a culture system and have catagorized it by: 1) radiofibril technique, 2) disc gel electrophoresis, 3) viscomistry. Further characterization of this enzyme and inhibition characteristics are currently underway.

Effect on collagen biosynthesis fibroblasts culture - In fibroblasts in culture the various factors affecting the rate of collagen synthesis are being examined. Primarily at this time the effect of pH on precursors of collagen biosynthesis and on the enzymes of collagen biosynthesis is being examined. This work is a basic approach to all cells in tissue culture and the effect of pH on their growth characteristics.

Significance to Cancer Research:

Tumor cells producing high amounts of collaginase have been shown to be exceedingly invasive. We now have a tool to study various tumor cells in tissue culture and determine their collagenolytic activity. It is possible by this tool to study the relative invasiveness of various carcinomas and investigate means for the inhibition of this phenomenon.

Proposed Course of Project: Ongoing

Honors and Awards: None

Publications: None

Serial No. NCI-3625
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Epidermal Macromolecular Synthesis in Newborn Rat, and Normal and Diseased Human Skin: An Electron Microscopic, Autoradiographical Study.

Previous Serial Number: Same

Principal Investigator: Gary Peck, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total: .05
Professional: .05
Others:

Project Description:

Objectives:

1) To investigate the factors controlling macromolecular biosynthesis.
2) To define certain dermatologic disorders at a biochemical level, by identifying which control factors are impaired.
3) Specifically, to study the incorporation in vitro of radioactive precursors into biopolymers in newborn rat, and normal and diseased human skin.

Material:

1) Whole newborn rat skin, cut into 3 mm 2 fragments
2) Trypsin separated rat epidermis
3) 0.1-0.2 mm thick, Castroviejo keratotome skin slices of normal and diseased human skin.
4) Isolated dermal fibroblasts, obtained from punch biopsies of human skin.
5) Isolated epidermal cells, obtained by dispersion after trypsin treatment of the keratotome slices.

Methods Employed:

Initially, fragments of newborn rat skin in culture media containing radioactive precursors were incubated at 37°C, for 6 hours, in a Dubnoff water bath shaker. At the end of the incubation specimens were taken for autoradiography; both light and electron microscopic, and for chemical extraction to measure the specific activity of labelled biopolymer.

Subsequently, the procedure was changed to a pulse-chase experiment, and diseased and uninvolved skin from a patient with Darier's disease was used.

Major Findings:

1) The in vitro incubation technique produced excellent autoradiographs, even as early as 2 weeks of emulsion exposure.

2) Initial localization of H^3-Histidine over the epidermal granular layer, and specifically over the keratohyalin granules, as reported by Fukuyama and Bernstein in newborn rat skin, was confirmed, extraction of keratohyalin granules and the H^3-histidine containing protein was performed with 1 molar phosphate buffer, at the suggestion of Dr. Arthur Ugel, who did the same experiment using fetal calf hoof.

3) H^3-Lysine H^3-Cystine, and ^3H Glycine gave diffuse labelling patterns over the epidermis in both involved and uninvolved skin. Histidine H^3-localized over the granular layer.

4) Much time was spent in the unsuccessful attempt to isolate rat epidermal cells in culture. The problems involved were a) contamination of cultures (as described in NCI-3630) and b) a high percentage of cell death resulting from trypsinization for dermal-epidermal separation.

5) In Darier's disease, preliminary data show that the specific activities obtained from incubating labelled H^3-Amino acids, histidine, lysine, and cystine with diseased skin were higher than those obtained from uninvolved skin. Specific activities obtained from H^3-Thymidine incorporation showed no difference between diseased and uninvolved skin. H^3-uridine labelled epidermal nuclei heavily-particularly in uninvolved skin, and at all levels of the epidermis. This may be interpreted as suggesting that there is a specific error in protein metabolism in Darier's disease. In psoriasis, protein synthesis is increased but it is thought to be secondary to accelerated cell turnover.

Proposed Course of Project:

1) Continue the study of Darier's disease to see if all or only a few amino acids show accelerated synthesis into protein. Darier's disease is responsive to high doses of Vitamin A. We may then repeat in vitro incubation studies in patients with Darier's disease after one month's treatment with high doses of Vitamin A to see if the accelerated protein synthesis is reversed.

2) It will be necessary to use normal volunteers for comparison: Application to obtain skin from normal volunteers has been approved.

3) At present isolation of rat epidermal cells is being attempted in tissue culture in preparation for *in vitro* labelling. Once this has been accomplished successfully, I will attempt this with human skin.

Significance to Cancer Research:

Knowledge of the Pathophysiology of hyperplastic disease states, such as Darier's disease and psoriasis, may lead to a greater understanding of the mechanisms involved which leads to cellular anaplasia and epidermal carcinoma.

Honors and Awards: None

Publications: None

Serial No. NCI-3626
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In Vitro Carcinogenesis Utilizing Dimethylbenzanthracene and Other Solvents.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Peter M. Elias, M.D., Stuart Yuspa, M.D., Richard Bates, M.D., and Marisa Gullino, M. Ph.

Cooperating Unit: Bio-Assay Section, NCI

Man Years (July 1, 1970 through June 30, 1971)

Total:	.9
Professional:	.4
Others:	.5

Project Description:

Objectives:

The original purpose of this study was to establish the nature of the cells undergoing malignant transformation in organ cultures of newborn mouse skin exposed to chemical carcinogens. Further studies are underway to determine the early morphologic events of carcinogenesis and the effects on cultured cells of chronic exposure to various solvents used as vehicles for carcinogens.

Methods Employed:

Cultures of transformed cells at various time periods following exposure to Dimethylbenzanthracene and various organic solvents, such as Tween 80, are fixed and embedded in situ for electron microscopy. Tumors that develop in isologous mice following injection of transformed cells subcutaneously are being studied ultrastructurally. Finally, these tumors are then re-suspended and cultured, and also processed for electron microscopy.

Major Findings:

We have identified the transforming cells in vitro as being epidermoid carcinomas by the presence of tonofibrils and desmosomes. There is no evidence of sarcomatous growth, although fibroblasts are undoubtedly introduced into the original culture. No evidence of viral contamination has been found on culture or ultrastructurally; thus, the transformation is almost certainly due to the chemical carcinogen. Another finding has been the tendency for very anaplastic squamous cell carcinomas to develop at the sites of inoculation. These cells are recognized as being of epidermal origin only by their rare desmosomes and scanty tonofibrillar material. Yet when re-introduced into tissue culture these cells become more differentiated, developing into epidermoid cells essentially identical to the original cultures.

Significance to Cancer Research:

As far as we are aware, all other attempts at in vitro carcinogenesis with virus or chemical agent have produced sarcomas. This is the first report of successful carcinogenesis in vitro. By studying the earliest morphological events in carcinogenesis, we hope to find a specific change which would enable biopsy material to be identified as malignant, pre-malignant, or hyperplastic.

Proposed Course of Project:

None

Honors and Awards: None

Publications: None

Serial No. NCI-3629
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Use of Osmium Zinc Iodide (O_sO_4 - ZNI_2) as a Special Stain for the Golgi Apparatus and Other Subcellular Organelles.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Peter M. Elias, M.D.

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:	.7
Professional:	.2
Others:	.5

Project Description:

Objectives:

To determine the pattern of organelle staining in hydras by variations of the O_sO_4 - ZNI_2 method.

Methods Employed:

All material is eventually studied by electron microscopy. Initially, the tissue is exposed for varying time periods to different concentrations and substituted combinations of O_sO_4 - ZNI_2, phosphate buffer, and other cationic metallic-substances; then, it is routinely processed for EM.

Major Findings:

Following 4 hours, combined fixation-staining with O_sO_4 - ZNI_2, the stain was limited to the Golgi apparatus and certain surface coats of Hydra littoralis and Hydra pseudoligactis. Adjustment of the staining solution to neutral pH with .INNaOH results in staining limited to the Golgi apparatus. Shorter or longer staining with O_sZI produces only partial Golgi staining and absence of surface coat staining.

Significance to Cancer Research:

Proposed Course of Project:

To be continued.

Honors and Awards: None

Publications: None

Serial No. NCI-3630
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Vitamin A Acid Induced Mucous Metaplasia of 13 day Embryonic Chick Metatarsal Skin in Organ Culture: An Histochemical and Electron Microscopic Study.

Previous Serial Number: Same

Principal Investigator: Gary Peck, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total:	1.0
Professional:	.4
Others:	.6

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To determine if epidermal cells are capable of both mucin and keratin production, or whether the two processes are mutually exclusive.

3) To study the early changes of Vitamin A acid induced mucous metaplasia at an ultrastructural level.

Material:

Metatarsal Shank Skin (devoid of feathers) is obtained from 13-14 day chick embryo.

Methods Employed:

Chick metatarsal skin is cultured in vitro and stimulated to undergo mucous metaplasia by Vitamin A acid at varying doses. Specimens are taken at varying time intervals and evaluated for mucin production by the use of special stains, such as PAS (with and without diastase), Alcian Blue, Toluidine Blue, and Colloidal iron. Specimens are also fixed in glutaraldehyde for subsequent examinations under the electron microscope.

Major Findings:

1) Using 30 international units of Vitamin A acid per milliliter of culture media, it was found that mucous metaplasia occurred within 2 days in in vitro incubation. Maximum metaplasia took place by 6 days.

As little as 1 I.U. produced metaplasia in 6 days.

We have studied specimens of chick skin harvested prior to 2 days of incubation and treated with low dosage Vitamin A in order to determine the earliest mechanism involved in mucus metaplasia. Ultrastructurally, we found no specific mechanism operative, other than increased amounts of rough endoplasmic reticulum and polyribosomes indicating increased protein synthetic activity.

2) Microscopically, the control specimens keratinized normally with the formation of thick bundles of tonofilaments, keratohyalin granules, and a multi-layered stratum corneum. Vitamin A acid treated specimens did not undergo keratinization. There was no tendency of the columnar basal cells to flatten as they approached the surface. Neither stratum corneum, nor keratohyalin granules were formed. Tonofilaments were present in small, fine bundles. In addition, there was increased folding of the nuclear membrane, and increased amounts of rough endoplasmic reticulum and polyribosomes. Mucin was present in the cytoplasm as amorphous droplets of electron dense material. These electron dense bodies appear similar to lysosomes; however, these structures are acid-phosphatase negative. In addition to mucin formation there seems to be a glandular metaplasia in the epidermis with formation of acini. This has not been demonstrated previously. Our standard system now utilizes 13-14 day old chick embryos treated with 20 i.u. Vitamin A acid in Eagle's Minimal Essential Media with 5% fetal calf serum. Cultures are harvested at 2-3 days.

3) Unfortunately, we have been hampered severely by a contamination problem. The usual techniques including sterile technique, adding antibiotics to the media, filtering the media, and adding silver nitrate to the water of our CO2 incubator have not controlled the contamination. We have been working with the Environmental Services Branch on this matter. We are hoping that the remodeling of our room to decrease the amount of traffic in the area, and the installation of a laminar flow hood, and some structural modifications in the design of our CO2 incubator to eliminate stagnant water traps will eliminate this problem. Many experiments have been lost when degenerative changes, not seen on light microscopy, were found at the ultrastructural level, and were due to mild contamination. Our main problem in this project has been the preservation, fixation, and preparation of satisfactory specimens for ultrastructural examination.

Significance to Cancer Research:

Knowledge of cell differentiation is basic to the understanding of malignant processes.

Proposed Courses of Project:

1) Continued ultrastructural examination of the above material.

2) Selective inhibitors such as actinomycin D, cycloheximide, and puromycin, are being tested to determine the site of action of vitamin A in this system.

3) The effect of Vitamin A acid on epidermal cell kinetics will be made by observing the incorporation of tritiated thymidine by chick metatarsal skin.

Honors and Awards: None

Publications: In preparation.

Serial No. NCI-3631
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Treatment of Skin Cancer with Topical BCNU, and CCNU.

Previous Serial Number: Same

Principal Investigator: Gary Peck, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total: .1
Professional: .1
Others:

Project Description:

Objectives:

To evaluate the therapeutic effectiveness and toxicity of BCNU and CCNU administered topically to the skin surface of teh above patients.

Agents:

0.1%, 0.2% and 0.4% Bis-Chloro-ethyl-nitrosourea; and 0.1%, 0.4% and 1.0% chloroethyl-nitrosourea; in 30% liquid petrolatum, 70% white petrolatum.

Material:

1) Basal Cell Carcinoma

2) Nevoid Basal Cell Carcinoma Syndrome

3) Xeroderma pigmentosum with cutaneous carcinoma.

Methods Employed:

1) Patients must have pathologic confirmation of the clinical diagnosis.

2) They must have normal hematologic, hepatic, and renal function.

3) Lesions on eyelids and canthi are not included.

4) Patients are cautioned to avoid sun.

5) Ointments are applied twice daily to affected areas for 8 weeks.

6) Laboratory tests are done before, during, and 6 weeks after therapy.

7) Serial photographs are taken.

Major Findings:

1) A total of 12 patients were included in this study. Nine received only BCNU ointment. One received only CCNU ointment; the remaining two received CCNU ointment initially and were given BCNU later as a second course of therapy. Five of the 12 patients were treatment failures by other modalities, including surgery and topical 5-FU in propylene glycol. Four of the patients had had X-ray therapy of their acne earlier in life. Four other patients had moderate to severe actinic skin. Two patients had the basal cell nevus syndrome; one had xeroderma pigmentosum. Most patients had lesions of the exposed areas of the face and neck. Many patients also had lesions on the trunk and extremities. None of the 12 had the standard variety of BCE histologically; the remaining three were superficial BCE, morphea type of BCE, and the fibroepithelioma type of BCE (Pinkus). There were nine males, three females, varying in age from 23-65.

2) Results of treatment are as follows:

BCNU	0.1%	26 lesions	10 cures	15 partial responses	one no response
CCNU	1.0%	7 lesions	1 cure	5 partial responses	one no response

Partial responses is defined as visible flattening of the tumor mass. The ten BCNU cures were in only four patients.

3) Severe contact dermatitis has occurred in some patients with 0.1% BCNU ointment. The dermatitis appeared from day 2 to day 33 in the different patients. Several patients have not developed any cutaneous irritation. In three patients tumors disappeared without antecedent skin inflammation. Usually the patients who had no skin reaction to the medication had little to no therapeutic response. This supports the clinical impression derived from experience with topical 5-FU: No inflammation, No Response.

4) The main difficulty in conducting this study was the lack of an assay system for the stability of the ointment. The active drugs are unstable in water or propylene glycol, therefore, petrolatum is the only suitable vehicle, and it is not possible to extract the active drug from the petrolatum ointment. This made establishing of the shelf-life difficult. By experience we determined that ointments over one month were not stable.

Significance to Cancer Research:

1) This is an exploratory study of a new drug in skin cancer prompted by a desire to find a superior agent to 5-Fluorouracil in propylene glycol which is in widespread topical use.

2) We would also hope to contribute to the knowledge of the clinical pharmacology of this agent. Skin cancer should prove to be a valuable lesion for the screening of this and future topical chemotherapeutic agents because the tumors are observable, measurable, and, therefore, can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

Proposed Course of Project:

1) Because of the variability in response to the drug and the lack of a stability assay for the ointment, this study has been discontinued.

2) Discontinuance of CCNU ointment as it produced partial responses in spite of prolonged therapeutic courses.

Honors and Awards: None

Publications: In preparation. Presented at the Psoriasis Club, Atlantic City, May 2, 1971 inconjunction with the Society for Investigative Dermatology.

Serial No. NCI-3632
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Toxic Epidermal Necrolysis and Graft-Versus-Host Reaction

Previous Serial Number: None

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: P. M. Elias, M. D., G. P. Herzig, M.D.

Cooperating Units: Medicine Branch, NCI

Man Years (July 1, 1970 through June 30, 1971)

Total: .05
Professional: .05
Other:

Project Description:

Objectives:

To investigate the relationship between Graft-Versus-Host Reaction (GVH) and Toxic Epidermal Necrolysis (TEN)

Methods Employed:

1) Taking of history, and repeated physical examinations of a patient with acute lymphocytic leukemia, treated with allogeneic bone marrow transplantation, who developed GVH, and subsequently, TEN.

2) Examination of multiple skin biopsies taken at various time intervals and at various skin sites.

3) Review of literature

Major Findings:

1) This is the first documented case in which the usual morbilliform eruption of GVH progressed in severity and led to a bullous eruption associated with sheet-like desquamation of the epidermis, typical of TEN.

2) Early in the clinical course, cutaneous histopathology was typical for GVH.

3) Later in the clinical course, biopsies taken from intensely erythematous sites, near areas of massive epidermal desquamation showed more extensive epidermal destruction, consistent with the diagnosis of TEN.

4) Support for our thesis linking TEN in this patient with his GVH, comes from animal studies in which experimental GVH in hamsters and monkeys developed TEN. Furthermore, a case of GVH was found in the literature in which a bullous eruption, in retrospect compatible with TEN, developed.

Significance to Cancer Research:

Immunosuppressed patients, including treated cancer patients, who are transfused or transplanted with immunocompetent cells, may expect to develop GVH. In view of this case report, we may now expect some of them to develop TEN as well.

Proposed Course of Project: Concluded

Honors and Awards:

First Prize - 1971 North American Clinical Dermatological Society Memorial Essay Contest.

Publications:

Peck, G. L., Herzig, G. P., and Elias, P. M.,: TEN in a patient with GVH Arch. Derm. In press. Presented at Annual Meeting, North American Clinical Dermatological Society, April, 1971, Cartagena, Columbia.

Serial No. NCI-3633
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Treatment of Psoriasis with Topical CCNU. (IND-7003)

Previous Serial Number: None

Principal Investigator: Gary Peck, M.D.

Other Investigators: Stephen Guss, M.D. and Douglas Key, M.D.

Cooperating Units: None

Man Years (July 1, 1970 through June 30, 1971)

Total: .15
Professional: .15
Others:

Project Description:

Objectives:

To evaluate safety and effectiveness of topical CCNU in psoriatic patients.

Agents:

0.4% and 1.0% CCNU in 30% liquid petrolatum and 70% white petrolatum.

Material:

Adult patients with psoriasis. Females must be past the childbearing age.

Methods Employed:

1) Patients must have psoriasis previously unresponsive to standard therapy.

2) They must have normal kidney, liver, and bone marrow function.

3) Medication will be applied twice daily until clear.

4) Patients will avoid sun exposure to prevent phototoxicity, if any.

5) Laboratory tests measuring bone marrow, liver, and kidney function will be obtained before, during, and 6 weeks after cessation of therapy.

6) Serial photographs will be obtained.

Major Findings:

1) Seven of 9 patients treated for longer than 2 weeks cleared completely. The 2 patients who did not clear had severe psoriasis of the palms and soles. Four additional patients were treated for less than 2 weeks.

2) Severe irritation of the skin surrounding the psoriatic plaques occurred. This was especially marked in the intertrigenous areas.

3) Subsequent to the cutaneous irritation, persistent pigmentation, telangiectasia, and cutaneous pain developed as well as temporary bone marrow suppression.

Significance to Cancer Research:

1) This study is part of an ongoing search for an effective topical therapy for psoriasis.

2) It is hoped that this study will contribute to the pharmacology of chemotherapeutic agents

Proposed Course of Project:

Because of the local and systemic toxicity, this study has been discontinued.

Honors and Awards: None

Publication: In preparation. Presented at the Psoriasis Club, Atlantic City, May 2, 1971 in conjunction with the Society for Investigative Dermatology.

Serial No. NCI-3634
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Role of DNA Repair Mechanisms in the Etiology of Cancer

Previous Serial Number: None

Principal Investigators: Marvin A. Lutzner, M.D.

Other Investigators: Peter G. Burk, M.D., Richard J. Brown, M.D., C. Wesley Dingman, M.D., Peter F. Swann, Ph.D., and M. Patricia Fisher.

Cooperating Units: Nucleic Acids Section, Chemistry Branch, Etiology, NCI.

Man Years (July 1, 1970 through June 30, 1971)

Total: .3
Professional: .3
Other:

Project Description:

Objectives:

To learn more regarding the role of DNA repair mechanisms in maintaining the integrity of the mammalian genome. In particular, to determine (a) whether there are correlations between the effectiveness of repair mechanisms and the resistance to tumorigenesis of different tissues, (b) whether chemical and physical carcinogens interfere with DNA repair, and (c) if they do, whether the mechanism of this effect is the damage inflicted on DNA directly or the damage to DNA repair enzymes or both.

Methods Employed:

We have started this project by attempting to find (if it exists) a difference in the effectiveness of DNA repair in normal human fibroblasts and lymphocytes and those from patients with xeroderma pigmentosum. (Evidence collected by others indicating that the first step in DNA repair of ultraviolet light induced damage is deficient in these patients is not entirely convincing.) Cells are grown in tissue culture, DNA is labeled with various appropriate radioactive precursor molecules, the cells are irradiated with ultraviolet light, and the DNA isolated at

different times thereafter. Evidence for the excision of the induced defect and the repair of the excised region is looked for. In vitro enzymatic assays may be employed at a later date if feasible. Similar studies are planned using carcinogenic alkylating agents administratered in vivo to susceptible and non-susceptible tissues as the means for introducing defects in DNA.

Major Findings:

This project has just recently been started and no major findings of significance have been forthcoming as yet.

Significance to Cancer Research:

Only within the last few years has the problem of DNA repair and its potential importance for confering resistance to mutagenesis (and possibly carcinogenesis) received much attention. It would appear that understanding these systems and their role in the overall economy of the cell would benefit many areas of biomedical research (especially those pertaining to genetic dysfunction) and aid in understanding the etiology of at least some forms of cancer (e.g., skin cancer).

Proposed Course of Project:

To pursue the goals outlined above in Objectives.

Honors and Awards: None

Publications: None

Serial No. NCI-3635
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Investigation of lysosomal activity in Epidermolysis Bullosa Simplex (EBS)

Previous Serial Number: None

Principal Investigator: Gary Peck, M.D.

Other Investigators: Ervin Epstein, Jr., M.D. and Peter Elias, M.D.

Cooperating units: None

Man Years (July 1, 1970 through June 31, 1971)

Total:	.7
Professional:	.3
Others:	.4

Project Description:

Objectives:

To investigate the pathogenesis of blister formation in EBS.

Material:

1) Skin from patients with EBS

2) Skin from patients with EB dystrophica, xeroderma pigmentosum, and normals, to serve as controls.

3) Mouse liver.

Methods Employed:

1) Biopsies from traumatized and non-traumatized uninvolved skin of patients with EBS are fixed in 6% GTA in phosphate buffer for routine ultrastructural examination.

2) Similar specimens fixed in 6% GTA in cacodylate buffer will be examined for acid phosphatase activity.

3) Control tissues will be processed similarly.

Major Findings:

1) Uninvolved, non-traumatized skin of EBS is abnormal in 2 respects:

a) there is occasional basal cell degeneration with marked intercellular edema, fragmentation of tonofilaments, and dissolution of other intracytoplasmic organelles.

b) there is increased melanin pigment transfer from melanocytes to keratinocytes with formation of giant membrane-bound melanosome complexes.

2) these melanosome complexes are acid phosphatase positive. This has been reported in the literature and these complexes have been called lysosomes. No other acid phosphatase positive organelles are seen in our specimens.

3) However, appropriate acid phosphatase controls are also positive (incubation with sodium fluoride, omitting the substrate B-glycerophosphate from the incubation mixture, and even incubation of the tissues in lead nitrate alone.)

4) Acid phosphatase reactions in xeroderma pigmentosum, EB dystrophica, and normal skin react similarly.

5) Under routine ultrastructural examination, xeroderma pigmentosum differs from EBS by not forming large melanosome complexes; EB dystrophica produces intradermal blisters, and, as such, there is no basal cell degeneration.

6) Results of mouse liver acid phosphatase reaction are currently being obtained.

Significance to Cancer Research:

1) Basic understanding of melanin physiology.

2) Elucidation of the significance of the acid phosphatase reaction in human skin.

3) Confirmation of published work on electron microscopy of EBS.

Proposed Course of Project:

1) Continued EM observations

2) Study the effect of topical Vitamin A acid on the EM findings and on the clinical disease.

Honors and Awards: None

Publication: In preparation

Serial No. NCI-3636
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Susceptibility of Fibroblasts from Xeroderma Pigmentosa Patients to Oncogenic Virus Transformation In Vitro.

Previous Serial Number: None

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Douglas Key, M.D., and Stuart Aaronson, M.D.

Cooperating Units: Viral Carcinogenesis Branch

Man Years (July 1, 1970 through June 30, 1971)

Total: .97
Professional: .97
Other:

Project Description:

Objectives:

Xeroderma pigmentosa is a genodermatosis with marked predisposition to development of carcinomas in sun exposed areas. An enzyme defect in DNA repair, post UV damage, has been shown in some of these patients. The purpose of this study is to obtain parameters of oncogenic virus infection and transformation of fibroblasts from normals and patients with xeroderma pigmentosa after exposure to varying dosage of ultraviolet radiation.

Methods Employed:

Fibroblasts as grown out from skin biopsy explants to undergo SV-40 transformation assay as described. Infectivity to be determined by immunofluorescent T antigen transformation by gross colony counts. Cell survival needed for obtaining the fraction of total colonies which are transformed will be determined by both thymidine-H^3 incorporation with liquid scintillation counting and by direct colony counts on feeder layers.

Major Findings:

Project initiated recently. No major findings yet.

Significance to Cancer Research:

The possible demonstration of enhanced susceptibility after UV irradiation to transformation by SV40 would be an exceedingly valuable *in vitro* model in view of: 1) the clinical predisposition to carcinogenesis in sun exposed areas in this rare genodermatosis; 2) Possible relationship to predisposition of older populations to sun-induced cutaneous malignancies.

Proposed Course of Project:

Exposure to varying dosages of UV radiation of fibroblasts of similar passage of both normals and xeroderma pigmentosa. Cell survival, infectivity, and transformation determined as described.

Honors and Awards: None

Publications: None

SUMMARY OUTLINE
Annual Report of the Immunology Branch, National Cancer Institute
July 1, 1970 through June 30, 1971

I. TUMOR IMMUNITY

- A. Immune Response
- B. Tumor Antigens

II. TRANSPLANTATION IMMUNOLOGY

- A. Bone Marrow Transplantation
  1. Man
  2. Monkey
  3. Mouse
- B. Histocompatibility Antigens
  1. Relation to Disease
  2. Purification and Characterization

III. CELLULAR IMMUNITY

- A. Recognition Units
- B. Immune Cells and Effector Systems

IV. REGULATION OF CELL GROWTH AND BIOSYNTHETIC ACTIVITY

- A. Human Lymphoid Cells
- B. Subcellular Systems

V. ANTIBODY MOLECULES AND IMMUNOGLOBULIN GENES

- A. Three-dimensional Structure
- B. Polypeptide Chain Composition

VI. IMMUNE DEFICIENCY AND ALLERGY

VII. DIAGNOSTIC AND CONSULTATIVE SERVICES

VIII. EDUCATION

- A. Professional
- B. Immunology Branch Staff

SUMMARY

Annual Report of the Immunology Branch, National Cancer Institute
July 1, 1970 through June 30, 1971

INTRODUCTION

In the past 7 years the Immunology Branch developed a capacity to investigate the immune system at the molecular, cellular, animal and human levels, and, in addition, apply this knowledge to diagnosis and treatment of disease in man. The early emphasis in the Branch was on the chemistry and immunology of antibodies and lymphoid and plasma cell tumors. This provided a sound base for extensions into cellular immunity, gene regulation of cell functions, and transplantation and tumor immunology. Today, the major emphasis is on the chemistry and immunology of tumor and normal cell surfaces and on the regulation and mechanisms (as well as the manipulation) of cellular and humoral immunity. A broad range of fundamental and clinical efforts are underway within the Branch as well as through contracts. The following review can only note some of the work being conducted in this extensive Program.

I. TUMOR IMMUNITY

A. Immune Response. Investigations in rats by Drs. Herberman and Ortiz de Landazuri revealed both cytotoxic antibody and cell mediated immune response to tumor specific (Gross virus) surface antigen. Suppressed cell-mediated immunity appears to be due to a central suppression of immune responses. Investigations are underway to see if this is a general phenomena.

Patients with acute leukemia were evaluated by Drs. Herberman and E. Rosenberg and others for evidence of anti-tumor immunity by three different methods. The results generally support the existence of cell-mediated immunity to antigens associated with leukemic cells.

Evaluation of tumor immunity in patients with solid tumors has been initiated by Drs. S. Rosenberg and Wunderlich, using lymphocyte migration and direct lymphocyte cytotoxicity assays respectively.

B. Tumor Antigens. Attempts to isolate tumor specific antigens have been initiated in rat Gross leukemia and human tumors by Dr. Herberman and associates. Relation of cell surface sialic acid to tumor specific antigens is being explored by Dr. S. Rosenberg. Dr. Ting has shown that appearance of SV-40 or polyoma antigens on transformed cells is associated with decrease in the normal H-2 antigens. These findings indicate that specific and profound changes in cell surface components accompany transformation by these oncogenic viruses.

Diagnostic service developments for tumor recognition in man are noted in section VII.

II. TRANSPLANTATION IMMUNOLOGY

A. Bone Marrow Transplantation.

1. Man. One transplant was performed in the Immunology Branch in a patient with aplastic anemia (Rogentine and associates) and 4 in acute leukemia by the Leukemia Service. Anti-lymphocyte serum (1 case) and cytoxan (4 cases) were used for immune suppression. Donor and recipients were HL-A identical. Of the four who had graft takes, one developed fatal graft versus host (GVH) disease, and several had minor GVH manifestations. Future studies will be directed at improved methods of preventing GVH disease.

A cytotoxic antibody against epithelial cells was found in several GVH patients (Merritt) indicating a possible mechanism for tissue damage in this syndrome. Immune functions and infection in GVH patients is under study.

2. Monkey. Typing reagents have been obtained for several leukocyte (? histocompatibility) antigens in the rhesus monkey. Bone marrow grafting success and prevention of GVH has been improved by using anti-lymphocyte serum. Detailed studies of significant factors in GVH disease are being developed in this model system.

3. Mouse. Efforts to prolong graft survival by administration of soluble antigen (E. Rosenberg, Mann) were successful. Present studies are directed at improving the desired effect and to determine if blocking antibody or tolerance is produced.

B. Histocompatibility Antigens.

1. Relation to Disease. A study of 47 patients with acute lymphoblastic leukemia (Rogentine) revealed a significant increase in the HL-A-2 antigen. Similar investigation in other neoplastic diseases are underway. Cell surface features such as HL-A antigens may influence the response to oncogenic agents or genes associated with alleles of the HL-A complex may determine susceptibility to malignancy by other means. Both biologic and chemical studies should help to answer these fundamental questions.

2. Purification and Characterization. New methods for solubilization and purification of HL-A antigens were explored and products with somewhat different properties were obtained. Most extensive studies have been conducted with papain solubilized antigen. Two molecular forms, differing in antigen specificities and apparently the product of separate genes within the HL-A complex, were identified.

HL-A specific antisera was induced in rabbits by purified HL-A antigen administration and appropriate absorption (Einstein). This is the first report of specific HL-A antisera induced in a heterologous species. Purified antigen is required for production of this antiserum as whole cells or crude membrane fractions introduce too broad a spectrum of antigens for the animal to mount an appreciable response to HL-A specificities.

III. CELLULAR IMMUNITY

A. Recognition Units. Immunologic theory indicates that the immune competent cell and the immune cell must have a recognition unit on the surface that is similar to antibody in specificity and, also probably in structure. A new assay for detecting antigen receptors on lymphocytes was developed by S. Rosenberg and preliminary studies indicate that the receptor contains immunoglobulin components but not sialic acid. Preliminary investigations revealed changes in cytoplasmic protein synthesis soon after exposure of immune lymphocytes to antigen. Future identification of the specific proteins and mechanisms of response to mitogens or antigens should provide insight into mechanism of gene activation in mammalian cells.

B. Immune Cells and Effector Systems. The relation between humoral and cellular factors in cell mediated cytotoxic reactions was studied. A serum antibody which causes non-immune lymphocytes to lyse target cells was identified by Wunderlich and E. Rosenberg. The lymphocyte dependent cytotoxic antibody (LDA) was identified first in the serum of a patient with chorio-carcinoma and is directed against the HL-A-7 antigen. Similar activity has been found in patients with auto-immune disease. The clinical significance of this new type of antibody is being explored.

In other studies, sensitization of lymphocytes so that they will react with specific target antigen was accomplished outside of the body in a mouse system by Wunderlich. This lead for direct induction of specific immunity is being developed further and possible application to man is under consideration.

IV. REGULATION OF CELLULAR GROWTH AND BIOSYNTHETIC ACTIVITY

A. Human Lymphoid Cells. Proliferation of human lymphoid cells in culture was shown to be limited by cell density (Buell and Fahey). During this stationary phase, the cell cycle was shown to be prolonged with the major change occuring in the G1 position of the cell cycle. Total protein, nucleic acid and immunoglobulin synthesis persisted during stationary phase at a reduced rate (in contrast to Log growth phases). Homeostatic mechanisms and soluble factors that influence this growth control are underway.

Changes in cell growth and function (immunoglobulin synthesis) when stationary cultures are reduced from high to low density are similar to the changes observed in antigen stimulated lymphoid tissue. Investigations of the cellular processes and factors that increase immunoglobulin formation (differentiation) and proliferation are planned.

A murine mouse model system (MOPC-315) which makes a myeloma protein with antibody activity has recently been established in tissue culture (Buell). In vitro and in vivo (BALB/c mice) studies with these cells are planned.

B. Subcellular Systems. Exploration of the molecular control of immunoglobulin synthesis in cell free systems was started with transplantable mouse plasmacytomas and with human lymphoid cells in tissue culture (Ein). Synthesis occurred on both membrane-bound and free ribosomes. However, a potent inhibitor activity effecting protein synthesis was identified on cytoplasmic membranes. The amount varied between plasmacytomas. The possible physiologic role of this factor(s) is being studied.

V. ANTIBODY MOLECULES AND IMMUNOGLOBULIN GENES

A. Three-dimensional Structure. A combination of three-dimensional and primary amino acid sequence information is required to understand the nature of the specific interaction between antibody and antigen. A program developing both approaches is beginning to bear fruit.

Terry, in collaboration with Davies and his associates (NIAID), constructed the first three-dimensional model of an immunoglobulin molecule. Further studies using X-ray crystallography are expected to provide even greater resolution.

Linear sequence information of the 4 polypeptide chains in the immunoglobulin crystal is being obtained, and extended to additional molecules with antibody activity.

B. Polypeptide Chain Composition. Heavy chains of different classes (γ, α and ε) were shown to have similar variable region sequences for the N-terminal amino acids. This finding indicates that the different heavy chain common region genes share a single pool of variable region genes.

Structural studies of Heavy chain disease proteins reveal them to be mutant forms of normal immunoglobulin heavy chains. Each of 4 examined in detail has been found to be distinctive. These proteins provide a unique opportunity to perceive how genetic material for antibody molecules is organized and expressed.

Homogeneous myeloma proteins with antibody activity and relatively homogeneous antibodies from immune animals are being secured for chemical study of the polypeptide chains and attempts to produce crystals for X-ray crystallographic studies.

VI. IMMUNE DEFICIENCY AND ALLERGY

Increased clinical admission of patients with immune deficiencies permitted characterization of a variety of disorders (Terry). Assays of cell-mediated and humoral immunity were established. Evaluation of (genetic) factors and reconstitution of function by transplantation of appropriate tissues are planned.

Studies of reaginic molecules are being performed with E-myeloma protein (Terry) to characterize the amino acid sequence of the epsilon-chain common

region. Quantitative assays for IgE, developed by Polmar, are being applied to a wide range of immune deficiency and allergic disorders.

VII. DIAGNOSTIC AND SONSULTATIVE SERVICES

Members of the Immunology Branch continued to provide consultation to physicians and investigators throughout the NIH and in the Washington area in connection with disorders of immunology, particularly of immunoglobulin formation and function, and in tissue HL-A typing.

The NCI Immunoglobulin Reference Center in collaboration with the WHO callibrated reference serums and is now distributing an international human serum immunoglobulin reference material to interested laboratories throughout the United States. In addition, the Immunoglobulin Reference Center during the past year tested about 1000 serums and urines for abnormal proteins. More than half were found to have abnormal proteins. In addition, aliquots of reagents capable of detecting specific human immunoglobulins were supplied to investigators through the country, including newly developed reagents for IgE.

New areas being developed at the NCI Immunoglobulin Reference Center include (1) assays for a number of antibodies in order to evaluate immune response in patients, and (2) immunodiagnostic tests for cancer. The first tests will be for human carcinoembryonic colon antigen.

VIII. EDUCATION

A. Professional. All senior members of the Immunology Branch continued to participate actively in education in Immunology and related sciences at the NIH. They planned and conducted lecture courses in the Foundation for Advanced Education in the Sciences, Tutorial Seminars in the Tutorial Program and working groups and research seminars within the Branch. They participate actively in the planning and conduct of NIH-wide continuing weekly seminars in fundamental immunology and in clinical immunology.

In the autumn of 1970, a lecture course at the graduate and postgraduate level was given by Terry (with others of the NIH) on Molecular and Cellular Aspects of Immunology to approximately 95 registrants. Tutorial Seminars were offered in Cellular Immunology by Terry, in Tumor Immunology by Herberman and Ting, and in Transplantation Immunology by Rogentine and Mann. An NIH-wide seminar series in Cell Biology was organized and conducted in January, 1971, by Fahey (with Marchesi, NIAID). The third annual Immunology workshop was planned and conducted in January 1971, by Terry in conjunction with Potter (NCI) and Metzger (NIAMD).

Members of the Branch gave over 50 lectures and seminars at other institutions throughout the country during the past year. More than 50 manuscripts were published and accepted for publication between July 1, 1970 and June 30, 1971.

B. Immunology Branch Staff. 17 persons in the Branch were supported in courses of education designed to improve their knowledge and capacity in biomedical science.

One person from the technical staff has already been accepted into medical school for autumn 1971, and another into the Harvard School of Public Health. In the past 5 years, 11 members of the technical staff have been accepted into Ph.D. or M.D. programs. Six of these are black and 5 are women.

Serial No. NCI-5000 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In Vitro Biosynthesis of Immunoglobulins

Previous Serial Number: Same

Principal Investigator: Daniel Ein, M.D.

Other Investigators: Marek Cwynarski, M.D., Stephen Polmar, M.D., Ph.D., and John L. Fahey, M.D.

Cooperating Units: None

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: The objectives of this project are to study the regulation of gene expression in mammalian cells using immunoglobulin synthesis as a model and to define defective regulation in mutant cells, i.e., cells from human individuals with a variety of primary and secondary immunoglobulin deficiencies.

Methods Employed: Subcellular particles from a mouse plasmacytoma and human lymphoid cells are separated by ultracentrifugal means. Soluble cellular components are fractionated by salting out, chromatographic and gel filtration techniques. Incubations of subcellular particles and soluble components are performed in the presence of radioactive amino acids. The products of in vitro synthesis are identified by immunochemical, polyacrylamide gel and amino acid sequence methods.

Two additional lines of investigation have been taken in the past year which use intact immunoglobulin-synthesizing cells. In one method, designed to study the initiation signals for immunoglobulin synthesis cells are incubated with a pulse of radioactive amino acids. The cells are fractionated, nascent peptides are isolated from polyribosomes and are characterized by gel-filtration and electrophoretic means.

Another study is in progress to investigate the rate of immunoglobulin synthesis by bone marrow cells of normal and immune-deficient individuals. Bone marrow cells are incubated for various time periods and both intracellular and extracellular fluids are assayed for immunoglobulin content by an inhibition of binding method.

Major Findings: The observations concerning a cell-free biosynthetic system reported last year for mouse plasmacytomas were extended to a system derived from a human lymphoid cell line in long-term tissue culture. It was found that both membrane-bound and free polyribosomes could be made to incorporate radioactive amino acids into acid-precipitable material. The energy and enzyme requirements for this system are the same as for those from mouse plasma cell tumors.

Further exploration of the mouse plasmacytoma system revealed that potent inhibitors of protein synthesis exist in membranes to which a large fraction of the cell's polyribosomes are normally bound. The most potent of these inhibitors appears to be an ATPase as previously described for microsomal membranes in other laboratories. A rather curious finding has been that the amount of this inhibitor varies greatly from one plasmacytoma to another. The amount of inhibitor is not clearly related to the class or quantity of immunoglobulin synthesized by the tumor and its physiological role is not known.

A major problem in all this work, limiting the usefulness of the in vitro system, has been that there is no consistent evidence of identifiable immunoglobulin synthesis. Occasional experiments revealed synthesis of heavy and light chains but these successes were only intermittent.

Two new projects are in early stages of development. Our investigations of initiation sequences for immunoglobulin chains has shown that two consistent small peptides containing Methionine (which might be the immunoglobulin initiator) are found on polyribosomes labeled with S^{35} Methionine. The rest of the amino acids in these peptides remains to be determined. It has been recently found that an antibiotic which blocks translocation of peptides from one ribosomal site to the next in cell-free systems, may be effective in whole plasma cells. This antibiotic has not previously been shown to have any effect on intact mammalian cells, reticulocytes in particular. Recent experiments in our laboratory suggest that high concentrations of this compound inhibit protein synthesis in plasma cells. The mechanism of inhibition is being investigated. The use of this substance under appropriate conditions should enable large quantities of initiating dipeptides to be synthesized thus making them amenable to amino acid analysis.

The second new project dealing with rates of synthesis of immunoglobulin-producing cells is still at the exploratory stage. Optimal conditions for culture of cells are being defined and the best method of assaying the immunoglobulin product is being investigated. Several assays have been tried including 1) direct binding of newly synthesized immunoglobulin antibody coupled to an insoluble adsorbent and 2) inhibition of binding radioiodinated

protein to antibody-Sepharose adsorbent. At the present time, synthesis of immunoglobulins by normal bone marrow cells can only be detected after 24 hours of culture but improved culture conditions and higher sensitivity of the assay should allow earlier detection of newly synthesized protein.

Significance to Biomedical Research and the Program of the Institute: The regulation of protein synthesis in mammalian cells is still a poorly understood set of chemical processes. Immunoglobulin-producing cells offer an excellent model for studying these events since each cell produces large quantities of a well characterized product and since the sophisticated means to identify and study immunoglobulin polypeptide chains are readily available in the Immunology Branch. There are theoretical considerations which suggest that the regulation of initiation of immunoglobulin synthesis differs from that of other proteins and clarification of this issue will amplify our understanding of either the universality or the complexities of the currently recognized initiation systems. There has been no previous investigation of the rates of immunoglobulin synthesis, on a per-cell basis, in hypogammaglobulinemic patients. Studies have shown diminished whole body synthesis of immunoglobulins and it is clear that many such patients suffer from a lack of immunoglobulin-producing cells. On the other hand, it may be that the defect for some patients lies in the rate of immunoglobulin synthesis by plasma cells and lymphocytes. If our studies show that protein synthesis is indeed impaired in these patients, then our understanding of these diseases will be much enhanced and work can proceed to define an exact molecular defect. A similar approach also seems reasonable in studying the causes of decreased antibody synthesis in acquired defects such as multiple myeloma and chronic lymphocytic leukemia.

Proposed Course of Project: 1) A return to further development of a cell-free system shall be made when deemed necessary for further understanding of the molecular defects in immune-deficiency states. 2) Initiating peptides in synthesis of immunoglobulins shall be further defined. 3) Rates of synthesis of various immunoglobulin by bone marrow cells from normal and hypogammaglobulinemic individuals shall be investigated.

Honors and Awards: None

Publications:

Ein, D. and Sox, H.C.: A cell-free protein synthetic system derived from human lymphoid cell lines in long-term culture. In preparation.

PHS--NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: NCI Immunoglobulin Reference Center

Previous Serial Number: Same

Principal Investigator: William D. Terry, M. D.

Other Investigators: Daniel Ein, M. D. and Elaine Esber, M. D.

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: The long range objectives of this project are to find and apply serum diagnostic tests for neoplasms in man. The immediate goals are to establish an NCI Immunoglobulin Reference Center in order to (1) improve immunochemical tests for multiple myeloma and macroglobulinemia by increasing the quality and availability of reagents, techniques and interpretation experience, (2) to facilitate studies of immunoglobulins by establishing national reference standards and by making available to qualified investigators small amounts of reagents that are not commercially available, (3) to investigate the relevance of serum immunoglobulin changes to disease prognosis and response to therapy, and (4) to assess myeloma serums for the capacity to bind various well defined antigens.

Methods Employed: Immunoelectrophoresis, employing a battery of specific antiserums, is used to detect specific immunoglobulin abnormalities. Quantitative immunochemical techniques, principally radial diffusion in agar plates containing antibody, are used to measure the serum levels of IgG, IgA, IgM and IgD as well as type K and type L immunoglobulins. Protein purification procedures (see Project NCI-5000) are used to prepare immunoglobulins for use as antigens to induce potent antiserums and as absorbents to obtain specific antiserums. Antiserums are produced in rabbits and larger animals. A modified Farr test using radiolabeled antigens is used to screen all sera for binding activity.

Major Findings: The principal effort on this project is conducted through contract PH 43-66-3. The NCI Immunoglobulin Reference Center has been established at Mel-Labs, Inc., in Springfield, Virginia.

Number of serums and urines tested 4/1/69 to 3/31/70	1,000
Number serums with anomalous immunoglobulins	394
Number of immunoelectrophoretic analyses performed	6,441
Number of Ouchterlony tests performed	1,000
Amount of specific antisera to human protein supplied to investigators	653 ml
Number of quantitative determinations of Ig concentrations	6,367

Large volumes of seven different antiserums are required for the diagnostic and quantitative studies. The Reference Center has immunized both small and large animals, collected the antiserums, evaluated them for strength and specificity and, where necessary, has absorbed them with appropriate sera or purified immunoglobulin preparations to render them useful for the immunodiagnostic work.

Immunoglobulin standards are needed, both at the Reference Center and at other institutions doing quantitative immunoglobulin studies throughout the country.

In collaboration with the World Health Organization Immunoglobulin Reference Center, pools of lyophilized plasma have been calibrated with purified proteins, and their stability characteristics evaluated. This lyophilized reference preparation is now available for distribution to any interested laboratory. The collaborative study to accurately calibrate the immunoglobulin content of the reference preparation is underway.

All sera shown to contain anomalous immunoglobulins have been tested for their ability to bind one of a number of radiolabeled haptenes, such as DNP-lysine. Sixteen sera containing M-proteins demonstrated significant binding activity for one or more of the haptenes. These sera are being investigated to determine whether it is the M-protein that has the binding activity.

Significance to Biomedical Research and the Program of the Institute: Clinicians concerned with diagnosis and care of patients and clinical pathologists concerned with laboratory testing have shown great interest in the specific immunochemical (immunoelectrophoretic) tests suitable for screening serum and urine for specific changes characteristic of multiple myeloma and Waldenstrom's macroglobulinemia. The tests eventually can be carried out throughout the country, but proper performance is required. The Immunoglobulin Reference Center has begun to provide a focus for materials and techniques.

The development of easily performed quantitative tests for immunoglobulins is accelerating research into the effects of disease, chemical therapy and other events on the individual components of the immunoglobulin system. The standardized procedures and high quality reagents at the NCI Immunoglobulin Reference Center provide reference materials and standards for immunoglobulin investigations in the United States.

The serums referred to the NCI Immunoglobulin Reference Center provide a valuable source of material for research conducted in projects on immunoglobulin structure (see Project NCI-5000), subclasses and genetic factors (see Project NCI-5002) and malignant diseases (see Project NCI-5005).

Proposed Course of Project: The NCI Immunoglobulin Reference Center will continue diagnostic work and production of needed reagents.

Reagents to identify a newly recognized immunoglobulin class, IgE, have been obtained and a search for patients with E-myeloma protein will be continued. One patient with this type of myeloma protein was identified this past year and the Immunoglobulin Reference Center will distribute some anti-E reagents that are now available.

Continued screening of myeloma sera for binding of DNP, as well as other antigens, will be carried out.

Two new areas are under development in this contract. Many patients with malignant diseases have evidence of deficient function of their immune systems. To help evaluate humoral immune function, the Immunoglobulin Reference Center has begun to establish antibody assays for a number of antigens. Dr. Elaine Esber has perfected techniques for assaying antibody activity to tetanus, diphtheria, blood group substances, pneumococcal polysaccharides, keyhole limpet hemocyanin and T2 bacteriophage. A technician has been hired and is being trained to perform these assays. When procedures are established, they will be made available to appropriate investigators for evaluation of patients.

The second area being developed is immunodiagnostic tests for cancer. The personnel at the Reference Center have good experience in isolating and purifying antigens and antibodies. The Reference Center is a logical place, therefore, to become a center for cancer immunodiagnostic procedures. It is planned to establish an assay for carcinoembryonic antigen and to make this the reference laboratory for a prospective study of the value of this test in the early diagnosis of various neoplasms.

Honors and Awards: None

Publications:

Fahey, J. L.: Developments in fundamental research that relate to clinical uses of immunoglobulins. In Merler, E. (Ed.): Symposium on Biologic Aspects and Clinical Uses of Immunoglobulins. Washington, D. C., National Academy of Sciences, 1970, pp. 15-30.

Terry, W. D.: Antibody activity of myeloma proteins. In Merler, E. (Ed.): Symposium on Biologic Aspects and Clinical Uses of Immunoglobulins. Washington, D. C., National Academy of Sciences, 1970, pp. 146-155.

Woods, R. and Hymes, F.: Report of NCI Immunoglobulin Reference Center. In Merler, E. (Ed.): Symposium on Biologic Aspects and Clinical Uses of Immunoglobulins. Washington, D. C., National Academy of Sciences, 1970, pp. 374-379.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genetic, Structural and Functional Characteristics of Human Immunoglobulins

Previous Serial Number: Same

Principal Investigator: William D. Terry, M.D.

Other Investigators: Thomas Waldmann, M.D., Stephen H. Polmar, M.D., Ph.D., and Daniel Ein, M.D.

Cooperating Units: Dr. David Davies, Laboratory of Molecular Biology, NIAMD
Dr. Henry Metzger, Laboratory of Arthritis, NIAMD
Dr. Shaul Kochwa, Mt. Sinai School of Medicine, New York
Dr. Murray Freedman, University of Toronto, Ontario, Canada

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives: The primary objectives of this project are to study the genetics and functional characteristics of human immunoglobulins and relate these characteristics to molecular structure. Finding previously unrecognized forms or categories of immunoglobulin molecules is an important part of this work. As different forms of immunoglobulin molecules are recognized, attempts are made to detect genetic factors in order that the genetic map of structural cistrons coding for immunoglobulin polypeptide chains can be more fully developed. In addition, aspects of the chemical heterogeneity of immune globulins are being studied to improve our understanding of the mechanisms generating the heterogeneity and to determine the possible biologic significance of this heterogeneity. The different forms of immunoglobulin molecules continue to be studied for correlations between molecular structure and biologic functions such as antibody activity, reaginic activity, etc. To further understanding of the functional significance of the classes and subclasses of immunoglobulins use will be made of "experiments of nature" leading to disease states characterized by selective immunoglobulin deficiencies. Patients with these diseases will be studied for functional aberrations of their immune systems.

Methods Employed: Studies required isolation of immunoglobulins from normal serum and also from the serum and urine of patients with multiple myeloma and macroglobulinemia. Isolation and analytic techniques included ion exchange chromatography, gel filtration, preparative electrophoresis, immune precipitation in gel, autoradiography and analytic ultracentrifugation. Protein structural studies utilized paper chromatography, high voltage electrophoresis the amino acid analyzer and the automatic amino acid sequencer. Ultracentrifugation, including both sedimentation and equilibrium techniques, has been used to characterize several systems.

Major Findings: The major findings of this project report are divided into a number of separate but related areas. 1) Three-dimensional structure of immunoglobulin molecules. Additional studies have been performed on the crystals of the IgG cryoprotein being studied with Dr. David Davies and his colleagues (Terry, Matthews and Davies, Nature 220: 239, 1968). Two different heavy atom derivatives have been obtained and have permitted the calculation of a model of IgG at a resolution of 6A°. A third heavy atom derivative is presently being used to permit calculation of a more precise model. Thus, the preliminary goal of this study has been accomplished and the first threee-dimensional model of an immunoglobulin molecule has been constructed..

This project is now moving in several directions. a) Attempts are being made to improve the quality of the crystals currently under study. b) Fc and Fab fragments of this molecule are being prepared and attempts will be made to crystallize these fragments. c) In collaboration with Dr. Richard Krause, Rockefeller University, attempts are being made to crystallize homogeneous rabbit IgG antibodies of known antibody specificity and if the whole molecules do not crystallize, to crystallize their Fab fragments.

2) Amino acid sequence of variable and common regions of immunoglobulin heavy chains. Determination of linear structure of selected portions of immunoglobulin molecules is important in providing data that can answer questions concerning the mechanisms for generating antibody variability and for biosynthesis of immunoglobulin polypeptide chains. Amino acid sequences of the amino-terminal portions of heavy polypeptide chains have been determined.

The results indicate that heavy chain variable regions can be divided into at least four major subgroups, based on sequence relatedness. A finding of extreme interest is that IgG, IgA and IgE molecules share these groups of variable region sequences. The immunoglobulin system has now been shown to contain three apparent pools of variable regions - one associated with κ chains, one with λ chains and one with all classes of heavy chains. Future studies of the variable region sequences of light and heavy chains of patients with malfunctions of the immune system may shed light on the functional significance of these variable region sequences.

Constant region sequences are being determined for IgE molecules. A collaborative study with Dr. Shaul Kochwa, Mt. Sinai Medical School, is designed to determine the comlete linear sequence of IgE constant regions. Preliminary findings include sequences around the first disulfide bridge.

Other properties of IgE are also under investigation. Several different quantitative assays for IgE have been established and tested by Dr. Stephen Polmar. Previously unrecognized artifacts leading to incorrect results have been discovered in two of the assays, making it possible to explain the variable results obtained in several different laboratories. An extensive survey of immune deficient patients as regards their levels of serum IgE is being carried out, in collaboration with Dr. Waldmann.

3) Structural characteristics of heavy chain disease proteins. These proteins are of interest because they represent mutant forms of immunoglobulin chains. As such, their structure should reflect the mutational event and possibly provide information concerning normal genetic control of immunoglobulins. Studies of one γ-heavy chain disease protein have shown that it has the carboxy and amino terminal amino acid sequences expected of γ chains. The molecular weight of this protein is lower than that of an intact γ chain, indicating that the protein is the product of a gene that has sustained an internal deletion. The genetic implications of single deletions affecting both common and variable portions of immunoglobulin chains are such that if there are separate genes for common and variable regions and if translocation is the mechanism whereby the genetic information is united, this must occur in the DNA and not the RNA.

Two additional heavy chain disease proteins have been studied. In both of these cases, the sequences begin in the middle of the γ-chain, at or near the region where papain and other enzymes cleave γ-chains. It is not possible to tell, therefore, whether these represent a different mutant form than previously reported heavy-chain disease proteins, or whether they have an N-terminal sequence that has been removed some time after synthesis. Attempts are being made to bring one of these patients to the NIH so that biosynthetic studies may be performed and the sequence of the synthesized product ascertained.

4) Antibody-like activity of myeloma proteins. A continued search for antibody activity in human myeloma proteins has been carried out at the NCI Immunoglobulin Reference Center (Serial # NCI-5001(c)). Sera from 350 patients have been studied for their capacity to bind 5 haptenes. Positive binding has been found in ten sera and these are presently under investigation to determine their antigen binding characteristics.

The third annual immunology workshop was planned in collaboration with Dr. Potter (NCI), Dr. Metzger (NIAMD). The workshop was devoted to homogeneous immunoglobulins with antibody-like binding activity, and to the general topic of autoimmunity. Support for the workshop was provided by NCI, NIAMD and NIAID and it was successfully held in early 1971 at the NIH. Plans are being made for another workshop next year.

5) The rationale for searching for homogeneous (myeloma) protein with antibody activity is based, in part, on the fact that conventional antibody is heterogeneous and thus not suitable for sequence or crystal studies. Recent observations (Freedman et al, in press) suggest that the heterogeneity of

conventional antibody is less than previously assumed. Freedman has suggested that isoelectric focusing can resolve at least some antibodies into a relatively small number of homogeneous components. A collaborative effort has been initiated with Dr. Freedman to obtain the amino acid sequences of the electro-focused fractions to determine a) whether they are structurally homogeneous and b) how many sequences may really be present in a single heterogeneous antibody preparation.

Significance to Biomedical Research and the Program of the Institute: The development of a more complete understanding of the operation and control of the immune system requires that the component parts of the system be identified as precisely as possible. In the past, this work has depended heavily upon the utilization of myeloma protein which can be isolated in a more homogeneous state than their normal counterparts in normal human serum. The finding that certain myeloma-like proteins both have antibody activity and form crystals under certain circumstances, leads to the exciting possibility of obtaining crystalline antibody molecules and of being able, therefore, to determine the three-dimensional structure of antibody molecules. It is only when both three-dimensional and primary sequence information can be put together that it will be possible to fully understand the nature of the interaction between antibodies and their antigens.

The generation of molecular variability among antibody molecules is not understood. Many theoretical formulations have been proposed and some of these can be subjected to critical testing. The accumulation of information concerning the primary amino acid sequences of the variable regions of heavy chains from the different classes and subclasses of immunoglobulin molecules should establish further constraints on the types of theories that may legitimately be entertained.

The relationship of immediate hypersensitivity to other immune mechanisms is poorly understood. The discovery of IgE myeloma proteins, associated with advances in the technology of cellular aspects of immediate hypersensitivity make meaningful investigation of this system a real possibility.

Proposed Course of Project: Continued search for antigenic, functional and genetic differences among the heavy and light polypeptide chains of human immunoglobulin molecules will be carried out.

Clinical investigations of selected patients with hypogammaglobulinemia and dysimmunoglobulinemia will be continued.

Structural correlates of antigenic, functional and genetic characteristics will be studied by determining amino acid composition, peptide map fingerprint analysis, carbohydrate analysis and, where indicated, amino acid sequences of appropriate immunoglobulin polypeptide chains.

Particular emphasis will be placed in the following areas: 1) Crystallization of various immunoglobulins. If a crystal of sufficient quality to give atomic resolution is obtained, the complete amino acid sequence of that protein will be determined. 2) The acquisition and study of myeloma proteins

with definable antibody activity. 3) The assessment of IgE-myeloma protein properties with an eye toward therapeutic implications.

Honors and Awards: None

Publications:

Steinberg, A.G., Terry, W.D., and Morell, A.R.: Human Allotypes and Genetic Dogma. Protides of the biological fluids. Ed. H. Peeters. Pergamon Press. 1970 p. 111-116.

Woods, R., Blumenschein, G.R., and Terry, W.D.: A New Type of Human Gamma Heavy Chain Disease Proteins: Immunochemical and Physical Characteristics. Immunochemistry, 7: 373-381, 1970.

Harrington, J.T., Hood, L., and Terry, W.D.: C-Terminal Peptides from Human γ-chains of Differing Subclass and Allotype. Immunochemistry, 7: 393-399, 1970.

Terry, W.D., and Ohms, J.J.: Implications of Heavy Chain Disease Protein Sequences for Multiple Gene Theories of Immunoglobulin Synthesis. Proc. Nat. Acad. Sci. (USA) 66: 558-563, 1970.

Ogawa, M., McIntyre, D.R., Ishizaka, K., Ishizaka T., Terry, W.D. and Waldmann, T.A.: Biologic Properties of E-Myeloma Proteins. Am. J. Medicine, in press.

Frommel, D., Litman, G.W., Terry, W.D., Good, R.A., Rosenberg, A: Conformational differences of immunoglobulin G subclasses. Biochimie et Biophysica Acta, 221: 399-402, 1970.

Terry, W.D., Ogawa, M., and Kochwa, S.: Structural Studies of Immunoglobulin E. II Amino Terminal Sequence of the Heavy Chain. J. Immunology, 105: 783-785, 1970.

Kaplan, A.P., Hood, L.E., Terry, W.D., and Metzger, H.: Amino Terminal Sequences of Human Immunoglobulin Heavy Chains. Immunochemistry, in press.

Sarma, V.R., Silverton, E.W., Davies, D.R. and Terry, W.D. The Three Dimensional Structure at 6A° Resolution of a Human γ G1 Immunoglobulin Molecule, J. Biol. Chem., in press.

Glenner, G.G., Terry, W.D., Harada, M., Isersky, C., and Page, D.: Amyloid Fibril Proteins: Proof of Homology with Immunoglobulin Light Chains by Amino Acid Sequence Analysis, Science, in press.

Davies, D.R., Sarma, R., Labow, L.W., Silverton, E., Segal, D. and Terry, W.D.: X-Ray Diffraction and Electron Microscope Studies on a Crystalline Human Immunoglobulin. Immunoglobulins, N.Y. Academy of Science, in press.

Kochwa, S., Terry, W.D., Capra, J.D., Yang, N.L., Structure of IgE Myeloma Protein, Immunoglobulins, N.Y. Academy of Science, in press.

Terry, W.D. and Ein, D.: Structural Studies of γ-Heavy Chain Disease Proteins, Immunoglobulins, N.Y. Academy of Science, in press.

Hood, L., Grant, J.A., and Sox, J.C., Jr.: In the structure of normal light chains from mammals and birds: Evolutionary and genetic implications. In Sterzl, J. and Riha, I. (Eds.): Developmental Aspects of Antibody Formation and Structure, Vol. I. Prague, Academia Publishing House of the Czechoslovak Academy of Science, 1970, pp. 283.

Hood, L., and Talmage, D.W.: On the mechanism of antibody diversity: A germ line basis for variability. Science 168: 325-334, 1970.

Hood, L.E., Potter, M., McKean, D.J.: Immunoglobulin Structure: Comparison of amino terminal sequences of kappa chains derived from genetically similar mice (BALB/c). Science, in press.

Grant, J.A., and Hood, L.: N-terminal analysis of normal immunoglobulin light chains - I A study of thirteen individual humans. Immunochemistry, Vol. 8, 63-79, 1971.

Serial No. NCI-5003 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cellular Immunity I

Previous Serial Number: Same

Principal Investigator: John R. Wunderlich, M.D.

Other Investigators: None

Cooperating Units: J. Martin, Ph.D., Laboratory of Immunology, NIAID, and J. Inman, M.D., Ph.D., Laboratory of Immunology, NIAID

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: The long range objectives of this project are: 1) to investigate the role of cellular and humoral factors in maintaining normal immune responses, 2) to define changes occurring in disease, 3) to manipulate these factors for therapeutic purposes. The immediate objectives of our work with a mouse model system have been 1) to further understand suppression of the cellular immune response by humoral antibody and 2) to develop means of enhancing the cellular immune response against syngeneic tumor cells.

Methods Employed: Cell mediated immunity is measured by the release of chromium^{51} from labeled target cells incubated with immune lymphoid cells. Immune cells are collected from spleen, marrow, lymph nodes, thymus, and peripheral blood. Target cells have been lymphoid cells from nodes, peripheral blood, tumors and tissue culture lines.

Major Findings: Last year we found that cellular immunity in mice is inhibited by hyperimmunization with allogeneic EL-4 tumor cells. Inhibition appeared to result from a feedback mechanism in which humoral antibody shuts off the development of cellular immune effector cells.

This concept has been solidified by our present findings. Inhibition due to hyperimmunization is specific for antigens used in hyperimmunization: 1) responsiveness to other antigens late in the course of hyperimmunization does not fall off; 2) passive administration only of antisera specific for the immunizing antigen inhibits cellular immunity early in the course of hyperimmunization. Inhibition due to hyperimmunization is apparent in all sampled parts of the lymphon: spleen, lymph nodes, bone marrow, thymus, and peripheral blood. Cellular immunity has been enhanced as much as 350-fold by transferring hyperimmunized lymphoid cells with fresh antigen into a permissive environment, such as an X-irradiated syngeneic host. Immunized hosts, however, do not provide satisfactory permissive environments. Blockage of cellular immune enhancement in these hosts is associated with preexisting humoral immunity. We thought that increasing the dose of antigen in hyperimmunized hosts might overcome antibody inhibition if it were a competitive type. However, inhibition has dominated over such attempts to increase cellular immunity in the suppressed hosts.

Recently, we have devoted time to preferentially selecting for a cellular immune response in previously unimmunized hosts. In collaboration with Dr. John Martin, and Dr. John Inman (NIAID), we have enhanced immunogenicity of syngeneic mouse tumor cells in the cellular immune response. This has been achieved by chemically coating X-irradiated tumor cells with either Conconavalin A or dinitrophenyl amino caproate prior to immunization. Subsequent host cellular immunity is measured against fresh untreated tumor. Increased cellular immunity appears to result from increased antigen processing by macrophages.

Bolstered cellular immunity associated with chemical coating of tumor cells most likely results from preferential development of cellular immunity. Immunization with uncoated tumor cells leads to a similar increase of cellular immunity if sensitized cells are handled in a permissive in vitro environment. In other words, immunization with chemically coated tumor cells appears to avoid development of a suppressive humoral response, or suppression is bypassed.

Significance to Biomedical Research and Programs of the Institute:
Our current findings offer both encouragement and warnings for tumor immunotherapy. Early in the course of disease, the immunogenicity of malignant cells may be increased by tumor cell alterations, such as coating them with new chemical determinants. At some point in the normal course of a malignancy, however, an anti-tumor humoral response may develop, which does not damage the tumor and ironically, suppresses the cellular immune response which might be able to reject the tumor. This suppressive humoral response will dominate over attempts to bolster a cellular immune response. Cellular immune activity can be 'rescued' from a suppressed state by cultivating sensitized lymphoid cells in a permissive in vitro environment. Such enriched anti-tumor cellular immune activity could prove therapeutically valuable if the suppressive humoral response in primary hosts were eliminated by X-irradiation or chemotherapy. Our techniques for enriching cellular immune effector cell activity also will be valuable for investigating the mechanisms of tumor cell destruction.

Proposed Course of Project: Work will be continued along three lines of investigation: Further understanding of 1) humoral antibody suppression of the cellular immune response, 2) mechanisms of target cell destruction by direct cell-mediated cytotoxicity, 3) enhancing immunogenicity of tumor cell antigens. Ongoing work concerning cellular immunity in humans is supported primarily by an NIH contract and is summarized in Project Report NCI-5016.

Honors and Awards: None

Publications:

Martin, W.J., Wunderlich, J.R., Fletcher, F., and Inman, J.K.: Enhanced immunigenicity of chemically-coated syngeneic tumor cells. Proc. Nat. Acad. Sci. 68: 469, 1971.

Serial No. NCI-5004 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Growth and Biosynthetic Activity of Cells of the Immune System

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M. D.

Other Investigators: Donald N. Buell, M. D.

Cooperating Units: None

Man Years:

Total:	4
Professional:	2
Others:	2

Project Description:

Objectives: 1) The development of large scale, long term cultures of functionally capable immune cells. 2) Understanding the mechanisms which regulate gene expression and the biosynthetic processes involved in the formation of antibodies and other immunoglobulins. 3) Delineate the factors which play a role in control of growth and proliferation of cells of the immune system.

Methods Employed: Tissue culture of human lymphoid cells is an important part of this work. Immunofluorescent cytology and radioimmunoelectrophoresis are used to identify the specific immunoglobulins produced. Rates of production and turnover are studied by continuous and pulse label incubations with radioactive amino acids, with analysis of incubation medium and cytoplasm using solid phase radioimmunoassay. SDS-acrylamide gel electrophoresis permits analysis of the synthesis and assembly of the subunit immunoglobulin chains. Standard counting techniques are used to follow cell growth. Incorporation of specific labeled precursors followed by autoradiography and scintillation counting are used to study rates of RNA and DNA synthesis, and to determine cell cycle parameters. Synchrony of cell growth is achieved by double thymidine and thymidine-colcemid blockade. The functional status of ribosomes is assessed after disruption of cells with detergent and cytoplasmic separation on ultracentrifugal density gradients.

Antibody secretion by individual cells of the functional murine myeloma MOPC-315 is measured by incorporating TNP coated sheep RBCs in a modified Jerne plaque assay. Hemagglutination with TNP-SRBCs is used to quantitate antibody levels in serum and ascites fluid.

Major Findings: Techniques were developed which permit IgG synthesizing IM9 cells to remain in a high density stationary phase with full viability for as long as 14 days. Under these conditions the cell cycle time was markedly increased and the G1 phase of the cycle was particularly prolonged to over 100 hours. Protein and immunoglobulin synthesis were reduced to near basal levels. These cells appear to be functionally equivalent to an in vivo population of resting lymphocytes which can be considered to be in a G0 or markedly prolonged G1 state.

IM9 cells were found to undergo a characteristic and reproducible transition from stationary phase to exponential growth during which they mimic changes seen in the activation of resting lymphocytes. Changes in DNA and RNA synthesis and alterations of cell cycle parameters were measured during transitional growth. Synchronous pulses of immunoglobulin production were seen, reminiscent of the synchronous events seen in the generation of antibody forming cells in early immune responses.

A useful murine myeloma model system (MOPC-315) has been developed which will permit further investigations of the interrelationships of cellular proliferation and immunoglobulin gene expression during plasma cell differentiation. An increased efficiency of antibody production can be seen with a slowing of tumor growth. This appears to be associated with cells spending a greater part of their time in the functional, immunoglobulin synthesizing G1 portion of the cell cycle. Most recently, several continuous cell lines of MOPC-315 have been established. These tissue culture lines remain tumorigenic and will permit parallel in vivo and in vitro manipulations.

Significance to Biomedical Research and the Program of the Institute:

1) Synchronization: An important development is the technique for inducing established tissue culture lymphocytes to enter a resting state. Large populations of these cells can then be induced to enter the cell cycle synchronously at a point in the G1 phase. This technique will prove useful to all workers interested in studying lymphocyte function.

2) Growth Control: A totally in vitro investigation of the repressor mechanisms involved in turning off lymphoid cell proliferation, and also mechanisms involved in lymphocyte activation, is now possible using tissue culture cell lines.

3) Myeloma: In vivo growth of a murine myeloma appears to follow a pattern nearly identical to other transplantable tumors. The alterations of immunoglobulin gene expression seen during growth of these malignant cells will provide important information towards understanding their counterparts, the normal antibody producing cells.

4) Immunoglobulin Production: Immunoglobulin production appears to be an integral part of the cell cycle of lymphocytes. Furthermore, immunoglobulin synthesis per cell increases as the number of cell divisions increases, suggesting that relahively stable factors that promote IG formation persist from one generation to the next in rapidly dividing populations. Investigation of such factors is now feasible.

Proposed Course of Project: Explore culture condition that will increase rate for successful establishment of human cells in permanent tissue culture. Factors such as CO_2, nutrients, feeder layers, etc., will be investigated.

Develop cell separation techniques in order to obtain specific functional cells for establishment in long term culture and detailed study.

Develop culture techniques for maintaining fully functional cells in high density cultures in order to establish high level in vitro antibody and immune cell production.

Investigate the specific molecular regulatory events involved in the onset of immunoglobulin synthesis in synchronized cultures.

Ribosomal function in log and stationary phases of culture growth will be compared as part of the investigation of factors determining specific protein production.

Lymphoid cell products which influence growth and proliferation of lymphoid cells will be sought.

Honors and Awards: None

Publications:

Buell, D. N., Sox, H. C., and Fahey, J. L.: Immunoglobulin production in proliferating lymphoid cells. In Cameron, I. L., Cameron, G. M., Zimmerman, A. M. (Eds.): Developmental Aspects of the Cell Cycle. New York, Academic Press, 1971, chapter 11.

Fahey, J.L., Buell, D.N., and Sox, H.C.: Proliferation and Differentiation of lymphoid cells: Studies with human lymphoid cell lines and immunoglobulin synthesis. Conference on Immunoglobulins, N.Y. Academy of Sciences, 1971, in press.

Serial No. NCI-5005 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Investigations of Malignant Lymphocytic and Plasmacytic Diseases

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M. D.

Other Investigators: Albert B. Einstein, Jr., M. D.

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Others:	0

Project Description:

Objectives: Malignant reticular and lymphomatous diseases are investigated with a view to determining the mechanisms responsible for the major disabling features of these illnesses, to clarify diagnosis and prognosis and to improve treatment. Investigations have focused on Waldenstrom's macroglobulinemia, multiple myeloma, heavy chain disease and related disorders with abnormal serum and urine immunoglobulins.

Methods Employed: Physicochemical and immunochemical techniques are employed for quantitative assessment of changes in immunoglobulins and other serum proteins. Quantitative immunologic techniques are used to measure response to antigenic stimulation. Histologic evaluation is conducted by histochemical, immunohistologic, electron microscopic and standard morphologic procedures. Clinical evaluation is based on specific protocols derived from previous experience.

Major Findings: Osteolytic lesions were found in six patients with macroglobulinemia. Contrary to common opinion, osteolytic lesions do not serve as an absolute distinction between multiple myeloma and macroglobulinemia. The features of these diseases are being reviewed and evaluated from the morphologic, immunologic and clinical standpoint.

A major review of 41 cases of macroglobulinemia seen by the senior investigators over a 15 year period is being conducted to synthesize information collected over this period about the many manifestations of this and related disorders.

Significance to Biomedical Research and the Program of the Institute: An appreciation of the diversity of disease manifestations and their relation to the abnormal serum protein, the response to therapy, clarification of factors determining prognosis, and the relative importance of hyperviscosity syndrome (and the need for plasmapheresis therapy) are being assessed.

Proposed Course of Project: Discontinuance due to departure of concerned investigators.

Honors and Awards: Meritorious Service Medal, USPHS, 1970.

Publications:

Fahey, J. L.: Function of the normal lymphocyte. In Holmes, W. L. (Ed.): Proceedings of Conference on Blood Cells as a Tissue. New York, N. Y., Plenum Press, 1970, pp. 187-199.

Fahey, J. L.: Developments in fundamental research that relate to clinical uses of immunoglobulins. In Merler, E. (Ed.): Symposium on Biologic Aspects and Clinical Uses of Immunoglobulins. Washington, D. C., National Academy of Sciences, 1970, pp. 15-30.

Fahey, J. L.: Cancer in the immune suppressed patient. Ann. Int. Med., in press.

Vermess, M., Pearson, K. D., Einstein, A. B., Jr., and Fahey, J. L.: Osseous manifestations of Waldenstrom's macroglobulinemia. Radiology, in press.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Transplantation Antigens

Previous Serial Number: Same

Principal Investigator: Dean L. Mann, M.D.

Other Investigators: Albert B. Einstein, Jr., M.D., Eugene B. Rosenberg, M.D., G. Nicholas Rogentine, Jr., M.D., John L. Fahey, M.D., Christopher C. Merritt, M.D., and William D. Terry, M.D.

Cooperating Units: Brigid G. Leventhal, M.D., Medicine Branch, NCI

Man Years:

Total:	4 1/2
Professional:	2 1/2
Others:	2

Project Description:

Objectives: The objectives of this study are: a) to purify human and monkey transplantation antigens; b) to chemically, genetically and immunologically characterize these antigens; c) to use purified transplantation antigens in biological tests to ascertain their ability to provoke homograft rejection or specific immunological tolerance; d) to study synthesis of cell membrane components bearing human transplantation antigens; e) use soluble HL-A alloantigens to raise allotypic antisera in heterologous species; f) to define and characterize cell surface components that function as vital receptor sites.

Methods Employed: ^{51}Cr release and dye exclusion cytotoxicity (See Project Report NCI-5008 were used to detect alloantigens. Human cells were obtained from normal blood bank donors and from tissue culture. Tumor cells, lymph nodes and spleen cells were used for assaying mouse H-2 alloantigens. Antisera detecting genetically determined human leukocyte alloantigens were obtained as described in Project NCI-5008. Mouse alloantisera were prepared in the conventional way. Alloantigens in subcellular form were detected by a cytotoxic inhibition assay using the ^{51}Cr release from target cells and employing the human leukocyte typing antiserums.

Human leukocyte alloantigens in a soluble form were prepared by graded hypotonic lysis of cell suspensions, recovery of lipoprotein membrane fragments

and papain digestion of these fragments to yield a water soluble antigen. Further purification was done by CM-Sephadex, gel filtration chromatography electrofocusing and acrylamide gel electrophoresis. H-2 antigens were prepared by essentially the same method by Stanley G. Nathenson, M.D., Albert Einstein College of Medicine, New York. A number of different techniques were employed to solubilize HL-A alloantigens. These included detergents, choatropic agents 3M KCl and sonication. The physical-chemical properties of the alloantigens were investigated.

In attempts to induce immunological tolerance to murine transplantation antigens, graded doses of the solubilized H-2^b alloantigens were administered intraperitoneally and intravenously to H-2^d sublethally irradiated mice and then challenged with tumors bearing the H-2^b histocompatibility antigns. Tumor growth and skin transplants were used to assess the tolerogenic effect of the soluble H-2 alloantigens.

To aid in studies of synthesis of HL-A alloantigens, heterologous antiserums were raised by injecting purified HL-A alloantigens into rabbits. These antiserums were evaluated for cytotoxicity to human lymphoid cells as well as for their ability to precipitate solubilized HL-A alloantigens. Synthesis studies were initiated by evaluating the optimal incorporation of radio-labeled amino acids into lymphoid cell membranes under various conditions of tissue culture. The antisera was absorbed with cells in attempts to raise specific allotypic antisera.

Major Findings: Preliminary studies of biologic activity of soluble HL-A alloantigens suggest that these materials are immunogenic, causing a decreased skin graft survival time. In-vitro soluble HL-A alloantigens induced blast transformation of lymphocytes from immunized individuals. These findings indicate that the soluble HL-A alloantigen preparations have biologic as well as serologic activity.

Heterologous antiserums to soluble HL-A antigens have been raised in rabbits. These antiserums are cytotoxic to a number of lymphoid tissue culture cell lines and human peripheral blood lymphocytes. These antiserums also precipitate solubilized HL-A alloantigens. After absorbtion the antisera were highly specific for individual HL-A alloantigens.

Prolongation of graft survival has been achieved in one system using soluble H-2 transplantation antigens. Prolonged survival of foreign H-2^b tissue (EL-4 tumors) was observed in BALB/c mice receiving 50 µgrams and 10 µgrams of the solubilized H-2^b alloantigens prior to tumor inoculation. This survival appears to be due to enhancing antibody. Further studies demonstrated prolonged survival of skin graft after soluble antigen administration and sublethal irradiation of the host. The mechanism appears to be enhancement.

New methods were explored to prepare soluble HL-A alloantigenic materials from lymphoid tissue culture cell sources. Chaotropic salts, detergents and urea were used to treat cell membrane preparation. The solubilized cell mem-

brane components were analyzed for HL-A alloantigenic activity by the method described.

The physical - chemical properties of materials solubilized by these methods were similar to the papain derived materials. These studies will aid in the determination of the molecular structure of HL-A alloantigens.

Soluble alloantigenic materials were prepared from monkey spleen cells, purified and characterized with their physical - chemical properties. These alloantigens have similar physical - chemical characteristics to the human (HL-A) transplantation antigens. The solubilized materials demonstrated serologic cross reactivity between the two species. These findings indicate a molecular relationship of the cell membrane antigens of these two related species.

Isolated Hela cell membrane components were used to immunize rabbits. Antisera raised to 2 components were cytotoxic to the original cell line. After appropriate absorptions with lymphoid cells the antiserum specifically blocked polio virus infection of the Hela cells. The evidence indicates that a specific membrane component functions as a receptor for viral infection. This work will be extended to the identification of this cell membrane component.

Significance to Biomedical Research and the Program of the Institute: The preparation and analysis of soluble human transplantation antigens has both fundamental and clinical significance. Basic information concerning the relationship of antigenic determinants to chemical structure will help in understanding the complex genetics of the HL-A locus. Clinically, soluble transplantation antigens may be used to induce specific tolerance to homografts and thus may obviate the need for hazardous immunosuppressive agents.

The participation of cell membranes in the control of cellular differentiation in normal growth and neoplastic transformation has not yet been well defined. Changes in membrane antigens either by modulation of present antigenic determinants or neoantigen formation have been demonstrated in some viral and chemically induced neoplasias. Chemical and structural characterization of membrane material from neoplastic cells can possibly provide information leading to immunotherapy and more appropriate chemotherapy of malignancies.

The ability to induce neoplasia in mice with viruses has been shown to be associated with the transplantation antigen phenotype. There are some preliminary studies in man that also suggest the HL-A antigens may be associated with certain neoplasms. The project designed to characterize the cell surface receptor for viral infection will be correlated with the HL-A alloantigen representation at the serological and chemical level. If a correlation is found, it would offer an explanation for the susceptibility of some individuals to viral infections (oncogenesis) and not others exposed to the same virus.

Proposed Course of Project: Further chemical and biologic characterization of soluble HL-A alloantigens will be undertaken. Specific studies

designed to correlate chemical structure and HL-A alloantigenic determinants are being pursued. The studies will extend to the chemical and biologic characterization of monkey transplantation antigens.

Preclinical testing of the pharmacology and toxicology of these preparations in animals and subsequent biological experimentation will be continued in patients. These biological experiments will be directed at answering two questions: a) are these antigens immunogenic and b) are they tolerogenic? If tolerance can be demonstrated, these antigens will be used in human hematopoietic cell transplantation (see Project Report NCI-5008) to ameliorate or prevent both graft rejection and graft versus host disease.

The heterologous antiserums raised against the soluble HL-A alloantigens will be tested for their potential as histocompatibility typing reagents. These antiserums will also be used to develop non-complement dependent assays for soluble HL-A alloantigens. These antiserums will be used in synthesis studies of the HL-A alloantigens in cell membranes. The antigens will be used to identify functional properties of cells, i.e. receptors for antigen recognition and viral infection.

Further biological studies relating to the induction and maintenance of tolerance with soluble mouse histocompatibility antigens will be undertaken.

Honors and Awards: None

Publications:

Nathenson, S.G., Shimada, A., Yamane, K., Muramatsu, T., Mann, D.L., Fahey, J.L., and Graff, R.G.: Biochemical properties of papain solubilized murine and human histocompatibility alloantigens. Federation Proceedings 29: 2026-2033, 1970.

Leventhal, B.D., Rogentine, G.N., Jr., and Mann, D.L.: Sensitization to water soluble transplantation antigens. Transplantation Proceedings, in press.

Mann, D.L., and Fahey, J.L.: Properties of HL-A alloantigens solubilized by chemical techniques. Transplantation Proceedings, in press.

Rosenberg, E.B., Mann, D.L., Hill, J.J., and Fahey, J.L.: Prolonged allograft survival in mice pretreated with soluble transplantation antigens. Transplantation, in press.

Merritt, C.B., Mann, D.L., and Rogentine, G.N., Jr.: Cytotoxic antibody in human graft versus host disease. Nature, in press.

Mann, D.L.: Comparison of HL-A alloantigen solubilized by papain and TIS. Advances in Immunol., in press.

Hollinshead, A., Herberman, R., and Mann, D.L.: Comparisons of HL-A allo-

antigens solubilized by papain and sonication. Proc. Soc. Exper. Biol. Med., in press.

Mann, D.L., Fahey, J.L.: Histocompatibility Antigens. Annual Review of Microbiology, in preparation.

Serial No. NCI-5008 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Transplantation of Hematopoietic Cells in Humans

Previous Serial Number: Same

Principal Investigator: G. Nicholas Rogentine, Jr., M.D.

Other Investigators: Christopher B. Merritt, M.D., John L. Fahey, M.D.

Cooperating Units: Edward Henderson, M.D., Brigid Leventhal, M.D., R.A. Yankee, M.D., Medicine Branch, NCI, and Robert Graw, M.D., Human Tumor Biology Branch, NCI; Paul Gerber, Ph.D., DBS; R.J. Trapani, Ph.D., Microbiological Associates, Inc.

Man Years:

Total	2
Professional:	1
Other:	1

Project Description:

Objectives: One of the primary objectives of these studies is the fundamental investigation of the nature and modification of transplantation immunity. A part of this objective is to test whether selection of histocompatible donor-recipient pairs will ensure successful bone marrow engraftment. This entails the establishment of reliable means of histocompatibility testing and a clinical transplantation program.

Another major objective is to identify and quantitate antigens of normal and neoplastic tissues with particular reference to HL-A transplantation antigens.

Methods Employed: Histocompatibility testing was carried out principally by studying genetically determined HL-A alloantigens on human lymphocytes. These antigens are assayed in vitro by a modification of the ^{51}Cr release lymphocyte cytotoxicity technique and the Terasaki microcytotoxicity test. Other tissue typing techniques such as dye exclusion cytotoxicity and quantitative complement fixation tests are available in the laboratory. All typing for transplantation is currently conducted under contract at Microbiological Associates, Inc., under the direction of Dr. R.J. Trapani.

HL-A antigens are detected by human alloantisera. We have 119 antisera recognizing twenty HL-A antigens in our panel at the present time. The source of these antisera has been described in Project Report NCI-5008, 1967-1968.

Human lymphoid cells growing in long term tissue culture were assayed both qualitatively and quantitatively for HL-A antigen content. These cells are maintained in the Immunology Branch laboratory and in the laboratory of Dr. Paul Gerber.

Major Findings: The relationship of histocompatibility matching to bone marrow engraftment - About 75 families and 1000 unrelated individuals were histocompatibility typed this year for possible bone marrow transplantation, or platelet or leukocyte transfusion. Several donor-recipient pairs were identified who were HL-A identical. This identity was confirmed by mixed leukocyte culture (done by Dr. Brigid Leventhal). Of this group of HL-A identical donors and recipients, five received bone marrow transplants after either large doses of cytoxan (four cases) or antilymphocyte globulin (one case). One of these patients, a young man with aplastic anemia, was on the Immunology Branch; and four, all with acute leukemia, were on the Leukemia Service, NCI. Four of five recipients had graft takes. Of the four, three had minimal or no graft versus host disease (GVH). The fourth developed fatal GVH. The success of engraftment and the absence of severe GVH in four of five cases demonstrates that matching for HL-A antigens is of vital importance to successful engraftment, and confirms similar findings reported last year (NCI #5008, 1969-1970).

The one failure of graft take was in a patient with acute myelogenous leukemia. Despite success in engraftment and avoidance of GVH, all of the four transplants were rejected and all patients except one are dead of the original disease.

The Bone Marrow Transplantation Group consisting of senior investigators from the Immunology, Human Tumor Biology, and Medicine Branches of NCI, continues to meet weekly to discuss transplant cases and pertinent experimental results.

The histocompatibility testing laboratory at Microbiological Associates, Inc., under the direction of Dr. R.J. Trapani is typing an average of 20 individuals' lymphocytes for HL-A antigens per day. The level of work from the contractor has been good and we anticipate a satisfactory future course. This facility does all the typing for marrow transplantation and the typing of blood bank donors and others for platelet and white cell transfusion (see Project Report of Ronald Yankee, M.D., Medicine Branch, NCI, 1971).

Immunopathology of GVH. The serum from the second case of severe GVH was found to have cytotoxic antibody for epidermal cells (see NCI-5008, 1970 for initial findings). The antibodies in all cases of GVH were found to be complement dependent IgM cytotoxins. Similar antibodies were found in sera of patients with lupus erythematosis, kindly provided by Dr. N. Talal, NIAMD, and not found in a variety of controls.

A significant effort began in our laboratory to identify qualitative changes in HL-A anitgens on cultured lymphoblasts following a variety of environmental changes. We have studied the HL-A antigens of three EBV infected lymphoblast cultures. Two of the three demonstrated no change in HL-A, a third demonstrated an apparent pick up of HL-A3 on the infected cell line. The cell line from which the infecting EBV was obtained was HL-A3 positive, also. We have further studied HL-A antigens on cytomegalovirus infected fibroblasts (with Drs. D. Fucillo and R. Henson, NINDS) and compared them to non-infected fibroblast lines. No qualitative differences were noted. Finally, we have studied with Dr. S. Rosenberg from the Immunology Branch, NCI, the effects of neuramidase treatment on the expression of HL-A antigens on lymphoblasts. There were no qualitative changes.

We have HL-A genotyped several more families with a variety of diseases, both malignant and non-malignant, in an attempt to determine if certain HL-A antigens or haplotypes were associated with the disease. These diseases included all types of leukemia, lymphoma, Hodgkins disease, aplastic anemia, immune deficiency syndromes, and familial cancer. Most of the data awaits analysis. However, 47 patients with acute lymphocytic leukemia have been analyzed. No one haplotype is present in excess frequency; all antigens we type for were present, but HL-A2 was significantly increased in gene frequency in this group of patients.

Significance to Biomedical Research and the Program of the Institute: Transplantation of hematopoietic cells in man is urgently needed as a means of correcting marrow aplasia due to chemical or physical agents. It is of particular relevance to other programs in the National Cancer Institute, as marrow aplasia is often seen in patients who have received high doses of chemotherapeutic agents or radiation for malignancy. The selection of compatible donor-recipient pairs is of prime importance in assuring graft acceptance and minimizing graft versus host disease. The success of the clinical marrow grafting program to date can be attributed in large part to proper selection of donor-recipient pairs.

One of the major problems in successful hematopoietic cell transplantation in man is the control of the graft versus host syndrome. While careful histocompatibility testing may prevent or reduce the severity of GVH, most needing marrow transplants will not have HL-A matched donors. Therefore, other investigations designed to prevent GVH are needed to overcome this stumbling block before marrow transplantation can be widely used as a treatment for marrow aplasia. The development of animal model systems is an essential element for preclinical trials of newer methods of suppressing GVH. Progress on these model systems is discussed in Project Report NCI-5011, 1971. When results from these animal model systems are available, they will be translated into clinical practice.

Cell surfaces are of importance in cellular recognition phenomena, cellular immunity and neoplasia. The HL-A antigen system is a convenient, easily assayed cell surface marker. Thus, studies of this antigen system on normal and neo-

plastic tissues may yield important basic and clinical information about neoplasia, cellular immunity and recognition phenomena. For example, susceptibility to the Gross leukemia virus is related to H-2 phenotype (H-2 is the murine leukocyte histocompatibility antigen locus analogous to HL-A). The finding that HL-A2 is present in greater frequency in patients with acute lymphocytic leukemia may be important in this regard. If the HL-A genotypes or phenotypes of man are associated with various leukemias, this may provide a means of identifying persons at risk from leukemia and may lead to more effective early treatment.

Proposed Course of Project: Patients with aplastic anemia, disseminated malignancies and immune deficiency syndromes will be admitted to the Immunology Branch for transplantation of hematopoietic cells. Bone marrow, fetal thymus and peripheral blood leukocytes will serve as sources of transplantable cells. Donors and recipients will be typed for leukocyte and erythrocyte antigens and compatible pairs will be selected. Transplantation may be performed in the absence of complete histocompatibility when sufficient animal model system data is available to suggest that new approaches to securing graft and preventing GVH will be successful in man.

Patients with various malignancies and other diseases will be typed for HL-A antigens to determine if certain phenotypes (and genotypes) are associated. Qualitative and quantitative studies of HL-A antigen content on normal and neoplastic leukocytes both in vivo and in tissue culture will be extended.

Honors and Awards: None

Publications:

Graw, R.G., Brown, J.A., Yankee, R.A., Widerlite, L.W., Leventhal, B.G., Whang-Peng, J., Rogentine, G.N., Jr., and Henderson, E.S.: Transplantation of HL-A identical bone marrow to patients with acute lymphocytic leukemia. Experimental Hematology 20: 35, 1970.

Oren, M.E., Herberman, R.B., and Rogentine, G.N., Jr.: Effect of heparin on the detection of human isoantigens by the cytotoxicity technique. Nature 225: 951, 1970.

Rogentine, G.N., Jr. and Gerber, P.: Qualitative and quantitative comparisons of HL-A antigens on different lymphoid cell types from the same individuals. In Histocompatibility Testing 1970, Munksgaard, Copenhagen, Terasaki, P. (ed.), p. 333.

Leventhal, B.G., Buell, D.N., Terasaki, P., Yankee, R.A., and Rogentine, G.N., Jr.: The mixed leukocyte response: Effect of maternal plasma. In Proceedings of the Fifth Leukocyte Culture Conference, Harris, J. (ed.), Academic Press, New York, to be published.

Leventhal, B.G., Mann, D.L., and Rogentine, G.N., Jr.: Sensitization to water soluble HL-A antigens. In Proceedings of the Third International Congress of the Transplantation Society, September 1970, in press.

Graw, R.G., Leventhal, B.G., Yankee, R.A., Rogentine, G.N., Jr., Whang-Peng, J., Hertzig, G.P., Halterman, R.H., and Henderson, E.S.: HL-A and MLC matched allogeneic bone marrow transplantation in patients with acute leukemia. In Proceedings of the Third International Congress of the Transplantation Society, September 1970, in press.

Graw, R.G., Brown, J.A., Yankee, R.A., Leventhal, B.G., Whang-Peng, J., Rogentine, G.N., Jr., and Henderson, E.S.: Transplantation of HL-A identical bone marrow to a patient with acute lymphocytic leukemia. Blood 36: 736, 1970.

Graw, R.G., Rogentine, G.N., Jr., Leventhal, B.G., Halterman, R.H., Berard, C., Herzig, G.P., Yankee, R.A., Whang-Peng, J., Krieger, G., and Henderson, E.S.: Graft versus host reaction complicating HL-A matched bone marrow transplantation. Lancet ii: 1053, 1970.

Rogentine, G.N., Jr., Rosenberg, S., Merritt, C.B., Yankee, R.A., Leventhal, B.G., Graw, R.G., Gripp, P., Whang-Peng, J., Fahey, J.L.: Successful allogeneic bone marrow transplantation in aplastic anemia. Exp. Hemat. 1970, in press.

Merritt, C.B., Mann, D.L., Rogentine, G.N., Jr.: Cytotoxic Antibody for Epithelial Cells in human graft versus host disease. Exp. Hematology, 1970, in press.

Serial No. NCI-5009 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immune Response to Tumor Specific and Other Cell Surface Antigens.

Previous Serial Number: Same

Principal Investigator: Ronald B. Herberman, M.D.

Other Investigators: Chou-Chik Ting, M.D., Manuel Ortiz de Landazuri, M.D., Joel Engelstein, M.D., Eugene Rosenberg, M.D., Steven Rosenberg, M.D.

Cooperating Units: Acute Leukemia Service, Medicine Branch, NCI; Laboratory of Cell Biology, NCI; Biology Branch, NCI; Viral Leukemia and Lymphoma Branch, NCI; Uganda Cancer Institute, Kampala, Uganda; George Washington University Contract #PH 692176; Bionetics Research Laboratories, Contract #PH 692160 and 70-2207; Dermatology Branch, NCI; Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden; Laboratory of Microbiology, NIDR

Man Years:

Total:	8
Professional:	5
Other:	2

Project Description:

Objectives: The objectives of this project are: 1) to detect tumor specific antigens and quantitative changes in normal antigens on malignant cells, 2) to detect antibodies and cellular immune response to tumor specific antigens, 3) to evaluate the diagnostic, prognostic and therapeutic significance of humoral and cellular immune response to tumor antigens, 4) to detect delayed hypersensitivity reactions and other immunologic reactions to human tumor antigens, and 5) to isolate and characterize tumor antigens and other cell surface antigens.

Methods Employed: Serum and cells are obtained from patients and control subjects. A variety of normal and tumor cell lines are maintained in tissue culture. Inbred strains of mice and rats, and transplantable murine tumors

induced by viral, chemical or unknown agents are used in model systems. Antigen-antibody reactions are measured by complement-dependent cytotoxicity reactions and by an indirect ^{125}I-labeled antibody technique. Quantitative levels of immunoglobulins are measured by radial immunodiffusion. Cellular immune reactions are measured by skin tests, mixed lymphocyte cultures, migration inhibition, lymphocyte-mediated cytotoxicity assay. Human and animal tumor and normal cell membrane and soluble fractions are prepared for antigenic and biochemical studies.

Major Findings:

I. Tumor Immunity in Animal Models:

a. The indirect ^{125}I-labeled antibody technique developed in our laboratory has proved useful in detection and study of antibodies to tumor specific cell surface antigens of tumors induced by polyoma virus (Ting and Herberman, 1970), by SV40 virus (Ting and Herberman, 1971) and by Gross leukemia virus. This has allowed quantitative studies of the antigenic content of various polyoma and SV40 tumors. It has been shown that the cell surface antigens detected by this assay, as opposed to reports with the less sensitive immunofluorescent assay, are closely correlated with the expression of tumor-specific transplantation antigens. The quantitative amounts of tumor specific cell surface antigens have been found to be inversely related to the quantities of H-2 histocompatibility antigens. Studies with this technique have not confirmed reports of uncovering of tumor cell surface antigens by treatment with trypsin or cross-reactivity of fetal antigens with tumor antigens.

b. The kinetics of the humoral (Herberman and Oren, 1971) and cellular (Oren et al, 1971) immune response to the Gross virus-induced cell surface antigen has been studied in a transplantable Gross leukemia in W/Fu rats. The cellular immune response was found to decline 20 days after tumor cell inoculation, concommitant with a rise in IgG antibody. Evidence indicates that serum antibody may suppress the cellular reactivity. Spleen cells, which on immediate testing have no cytotoxic activity, become strongly positive after in vitro incubation for 12-24 hours.

c. The lymphocyte mediated cytotoxicity assay has also been successfully used to study the cellular immune response to inoculation with Moloney sarcoma virus. Activity has been found 15-20 days after virus inoculation.

d. Plasma cell tumors in BALB/c mice have been shown to possess tumor specific transplantation antigens, which are common to several of the tumors. A naturally occurring cytotoxic antibody has been found in the sera of BALB/c mice which reacts specifically with the plasma cell tumors. The antigen detected by this reaction has many of the characteristics of, and is probably identical to, the PC-1 antigen described by investigators at Sloan-Kettering.

II. Tumor Immunity in Man:

a. Patients with neoplastic disease have been shown to have delayed hypersensitivity reactions upon inoculation with extracts of tumor cells. Studies performed with Drs. Avrum Blumbing and John Ziegler (Uganda Cancer Institute) on patients with Burkitt's lymphoma have indicated that reactivity correlates well with the clinical state of the patients. Reactions were positive in about 50% of patients in remission; these patients remained in remission for a significantly longer period of time than those with negative tests. Extensive skin testing on patients at the Clinical Center with acute leukemia has shown a similar pattern. Positive reactions were seen more often in remission than in relapse. Studies have been performed with Drs. C. Alford and A. Hollinshed (George Washington University) on patients with carcinoma of the colon and breast and with melanoma. Soluble fractions of colon tumors which produced positive skin reactions were found to contain the carcino-embryonic antigen of Gold (Hollinshead et al, 1970). A tissue culture cell line has been found which contains CEA and produces positive skin reactions. Skin reactions in patients with carcinoma of the breast have been elicited by extracts from tumor and from normal breast. It appears that tissue specific rather than tumor specific reactivity is being detected.

b. In a cooperative study with Dr. Brigid Leventhal and others (Acute Leukemia Service, NCI) patients with acute leukemia have been studied by three assays for cellular immunity: delayed hypersensitivity skin tests, mixed lymphocyte cultures, and lymphocyte-mediated cytotoxicity. The two in vitro tests correlated with each other 70% of the time and correlated poorly with the skin tests. Only the skin tests correlated with the clinical state of the patients. In all, eighty percent of the patients were shown to have reactivity with leukemia associated antigens.

c. A study of leukemia in identical twins has been performed. The lymphocyte cytotoxicity reaction has detected antigens on the cells of the patient with leukemia which could not be detected on the cells of the normal identical twin. Family members and some controls have shown positive reactions, raising the possibility of association of reactivity with exposure to an environmental agent, such as a virus.

d. Tumor cell extracts have been shown to cause inhibition of migration of the peripheral leukocytes of some cancer patients.

III. Tumor Specific and other Cell Surface Antigens:

a. The Gross leukemia virus-induced cell surface antigen was studied, by use of a cytotoxic inhibition assay. This antigen was found in the sera of animals bearing a transplantable Gross tumor. The antigenic activity was found to be associated with membranous material in the sera rather than with the Gross leukemia virus itself. Large amounts of the antigen could be extracted from tumor cells by repeated washing with saline. The antigen has been found to be more labile than H-2 antigen, and this has slowed the attempts at purification.

b. Masking of tumor-specific antigens by sialic acid has been postulated. Treatment of Rauscher leukemia virus induced tumor cells with neuraminidase has resulted in a 3-4 fold increase in detectable tumor specific surface antigen. Similar treatment of polyoma virus-induced tumor cells has not resulted in a detectable increase in antigen.

Significance to Biomedical Research and the Program of the Institute: Detection of tumor antigens and of antibodies to tumor specific antigens is essential for progress in tumor immunology. The sensitive, indirect ^{125}I-labeled antibody technique has already proved useful in detection of antibodies to mouse and rat tumor specific antigens. It has the potential of detecting antibodies to other tumor antigens, including those on human tumors.

The capacity to measure various aspects of the immune response to animal and human tumors has been developed in our laboratory. This should allow detailed dissection of the role of the immune response in limiting or preventing tumor growth. These assays will also be invaluable in measuring changes in tumor specific immunity, in response to immunotherapeutic procedures.

The available immunologic techniques have also allowed progress in the solubilization and isolation of tumor-specific cell surface antigens. Such studies will permit a comparison of the physicochemical nature of the tumor antigens and normal histocompatibility antigens and may provide material for active immunization of animals and tumor patients against their tumors.

Proposed Course of Project: The radiolabeled antibody technique will be further applied to the study of polyoma and SV40 tumor antigens. The relationship of this antigen to expression of viral gene functions will be studied. This assay will also be used to study antigens on human tumor cells, particularly acute lymphocytic leukemia.

The studies on the immune response to Gross virus-induced tumors will be continued. The emphasis will be on the effects of antibody on the cellular immune response. Studies on the nature and distribution of the cytotoxic cells will be performed. Immunotherapy experiments will be performed with antibodies, with immune cells, and with immunization with soluble Gross cell surface antigen.

Further studies on the relationship of tumor antigens to fetal antigens will be performed. Evidence for such cross-reactivity will be looked for in the various animal tumor systems. Studies will be performed to determine whether the carcinoembryonic antigen of Gold is responsible for the delayed hypersensitivity reactions in patients with carcinoma of the colon.

Clinical studies in tumor immunology will be continued. Patients will be tested serially with a battery of specific humoral and cellular immunologic assays. Patients will be injected intradermally with X-irradiated tumor cells or with soluble antigenic fractions. The effects of inoculation on the immunologic parameters will be studied. This information should prove invaluable for the design of rational immunotherapy trials.

Honors and Awards: None

Publications:

Ting, C.C. and Herberman, R.B.: Kinetics of the response of different classes of antibodies to skin allografts in mice. Transplantation, 11:390-395, 1971.

Herberman, R.B. and Oren, M.E.: Immune response to Gross virus-induced lymphoma. I. Kinetics of cytotoxic antibody response. J. Nat. Cancer Inst., 46: 391, 1971.

Oren, M.D., Herberman, R.B., and Canty, T.G.: Immune response to Gross virus-induced lymphoma. II, Kinetics of the cellular immune response. J. Nat. Cancer Inst., 46: 621, 1971.

Oren, M. and Herberman, R.: Delayed cutaneous hypersensitivity reactions to membrane extracts of human tumour cells. Clin. Exper. Immunol. (in press), 1971.

Herberman, R.B., Oren, M.E., Rogentine, G.N., Jr., and Fahey, J.L.: Cytolytic effects of alloantiserum in patients with lymphoproliferative disorders. Cancer (in press), 1971.

Ting, C.C. and Herberman, R.B.: Detection of tumor specific cell surface antigen of simian virus 40-induced tumors by the isotopic antiglobulin technique. Int. J. Cancer (in press), 1971.

Ting, C.C., Lavrin, D.H., Shiu, G., and Herberman, R.B.: Tumor specific cell surface antigens in papova-virus tumors and their relationship to fetal antigens. Oak Ridge Conference on Cancer and Fetal Antigens (in press), 1971.

Herberman, R.B., Hollinshead, A. and Alford, C.A.: Skin reactive soluble antigen from intestinal cancer cell membranes and relationship to carcino-embryonic antigens. Oak Ridge Conference on Cancer and Fetal Antigens (in press), 1971.

Ting, C.C. and Herberman, R.B.: Inverse relationship of tumor specific cell surface antigens and mouse histocompatibility antigens in polyoma virus-induced tumors. Nature (in press), 1971.

Herberman, R.B. and Nam, J.M.: Cytotoxic antibody reactive with culture of lymphoid cells: Occurrence in disease and in normal human sera. J. Nat. Cancer Inst. (in press), 1971.

Serial No. NCI-5011 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Basic Immunological Studies in Hematopoietic Cell Transplantion.

Previous Serial Number: Same

Principal Investigator: G. Nicholas Rogentine, Jr., M.D.

Other Investigators: Christopher B. Merritt, M.D., and John L. Fahey, M.D.

Cooperating Units: C. C. Darrow, D.V.M., Bionetics Research Laboratories, Inc.; G. Herzig, M.D., Medicine Branch, NCI.

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives: The objectives of this study are to investigate the central problem of transplantation immunology - the recognition and response to major and minor histocompatibility antigens - and to develop means to modify these processes. These studies are being carried out in rhesus monkeys undergoing bone marrow transplants and in which the graft versus host (GVH) reaction serves as an excellent model for investigation of all aspects of transplantation immunology.

An important part of these studies is the identification of the transplantation antigen system of the animal species used, the Rhesus monkey.

Methods Employed: Experiments done in the past 12 months have employed a variety of techniques and methods. Femoral bone marrow obtained by aspiration from anesthesized donors is administered intravenously to lethally X-irradiated recipients. Such allografted monkeys are then medically supported and observed for the remainder of their life with frequent measurement of a number of clinical, biochemical, and immunologic parameters. In some cases frozen autologous marrow has been given. Lymphocyte antigens of the Rhesus monkey are studied by standard trypan blue dye lymphocytotoxicity tests

employing Rhesus alloantisera obtained from planned immunizations with skin grafts and leukocyte injections.

Rhesus spleens obtained at operation have been used to prepare soluble lymphocyte antigens. Spleen cell membranes are digested by papain and purified by column chromatography and gel electrophoresis. Soluble antigens prepared in this manner are assayed in vitro for their ability to inhibit antisera against rhesus lymphocytes, and in vivo for their ability to cause accelerated rejection of rhesus skin allografts.

The production of anti-rhesus lymphocyte sera has continued with the immunization of rabbits and a horse with rhesus thoracic duct lymphocytes. The ALS resulting from these immunizations has been tested for activity in vitro, and for immunosuppressive properties in vivo using rhesus skin graft and GVH systems.

Major Findings: Modification of GVH in the rhesus monkey: In the past year we have been able to consistently engraft lethally irradiated rhesus monkeys with either autologous or allogeneic bone marrow. In the latter case we regularly see an acute GVH reaction. The mean survival time (MST) for these allografted animals with acute GVH is 9.1 days (10 animals). We have been successful in increasing the MST in allografted animals by pretreating them with horse or rabbit anti-human or anti-rhesus lymphocyte serum just prior to irradiation. In our initial experiments in 8 rhesus using weak ALS preparations we increased allograft survival to 17.8 days and saw a conversion of the clinical picture of acute GVH normally seen in allografted animals to a milder subacute form. We are presently evaluating a more potent rabbit anti-rhesus thoracic duct lymphocyte serum and have increased the MST in three allografted rhesus treated with this reagent to greater than 35 days. These results indicate a significant advance in our ability to modify the processes resulting in GVH. This system will be of great value in the investigation of the mechanisms involved in the GVH process.

In one monkey we have been able to completely control the problem of GVH by infusing autologous bone marrow cells 24 hours after giving the allograft.

Three rhesus monkey lymphocyte alloantigen groups have been defined by the cytotoxicity technique. Typing of 115 unrelated monkeys and 21 pedigreed families indicates that two antigen groups appear to be a part of a multiple allelic system, while the third is part of a separate but closely linked genetic system. These alloantigen groups may be the rhesus counterpart of the human HL-A alloantigens.

Soluble rhesus lymphocyte antigens have been studied with particular reference to their similarities and differences to the human HL-A antigens. Although rhesus lymphocytes react nonspecifically with HL-A typing reagents, immunochemical tests indicate that human and rhesus lymphocyte antigens in soluble form may be quite similar. A total of 7 rhesus soluble antigen prep-

arations have been made and have demonstrated specific activity in vitro. These antigens are now ready for further testing in vivo in efforts to induce tolerance and/or blocking antibodies.

Significance to Biomedical Research and the Program of the Institute: Research on the recognition and reaction to major and minor histocompatibility antigens is of fundamental importance in transplantation immunology. Furthermore, it has vital clinical importance. The National Cancer Institute has made a strong commitment to a clinical program of marrow transplantation. The first stage of this program is a reality. However, only HL-A matched donors have been used. This greatly limits the application of this form of therapy, as well over 60% of all potential recipients do not have matched donors. Transplantation of mismatched marrow has almost invariably resulted either in no graft take or fatal GvH, and even in perfectly matched donor-recipient combinations, fatal FVH may occur, although at a lower frequency. Our recent findings with respect to the modification of GVH with ALS provides a tool for study of the basic mechanism of GVH, and also may be applicable clinically, particularly in situations where it is necessary to transplant marrow between individuals not perfectly matched at HL-A. An understanding of the pathophysiology of GVH obtained in this system will also be of benefit in planning new methods of intervention in this problem as it occurs in man.

Proposed Course of Project: With respect to basic questions in transplantation, we will attempt to understand the pathophysiology of GVH, as well as the mechanism by which ALS acts to modify the GVH reaction. This will involve a number of observations comparing clinical, histopathological, and immunological data from ALS treated and untreated animals. Other methods will be used, either alone or in combination, in an effort to further control the GVH reaction. These will include further studies with soluble lymphocyte antigens in attempts to produce tolerance, and the use of blocking antibody to modify the GVH reaction. The possibility of using nonlethal forms of immunosuppression to prepare the recipient for transplant will also be considered (such as the use of ALS and sublethal doses of X-ray).

Further studies of monkey leukocyte antigens will be conducted using lymphocytotoxicity tests. New antisera will be produced by cross immunizing antigen matched monkeys. Special emphasis will be placed upon typing of families. Mixed leukocyte cultures will also be carried out in these families. The relationship of leukocyte antigens to graft success will be studied.

Honors and Awards: None

Publications:

Rogentine, G. N., Jr., Vaal, L., Ellis, E., and Darrow, C. C.: Hematological changes following allogeneic marrow grafting in the Rhesus monkey. A peripheral blood manifestation of graft versus host disese? Experimental Hematology, 20: 51, 1970.

Serial No. NCI-5012 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study and Reconstitution of Patients with Deficient Immune Systems

Previous Serial Number: Same

Principal Investigator: William D. Terry, M.D.

Other Investigators: G. Nicholas Rogentine, Jr., M.D., Daniel Ein, M.D., Stephen H. Polmar, M.D., Ph.D., and Elaine Esber, M.D.

Cooperating Units: None

Man Years:

Total:	2-1/2
Professional:	1 1/2
Others:	1

Project Description:

Objectives: Studies of the immune systems of experimental animals and of humans with various forms of immune deficiency has provided insight into the general categories of cells that function together in an immune competent individual. The information gained has led to preliminary successful attempts at reconstitution of deficient individuals by transplanting appropriate tissues from closely related, histocompatible donors. The objectives of the present study will be: 1) to identify families likely to produce offspring with these diseases, and to identify infants with nonfamilial forms; 2) to fully evaluate the immune parameters of these infants; 3) to attempt reconstitution by transplantation of thymic, bone marrow or other tissues; and 4) to follow the long-term effects of such reconstitution.

Methods Employed: Patients will be evaluated in terms of cellular and humoral immunity. Cells will be tested for their ability to respond with increased tritiated thymidine incorporation to mitogens, antigens and mixed leukocyte culture. In vivo delayed hypersensitivity will be tested by active sensitization with dinitrochlorobenzene. Additional skin tests for delayed hypersensitivity will be performed. Humoral immune capacity will be tested by stimulation with appropriate antigens. Histocompatibility antigens will be determined for all recipients and potential donors.

Major Findings: Graft versus host (GVH) disease is a major problem in transplantation of immunocompetent cells. A model system is being studied to explore methods for modifying or avoiding GVH reactions. Histoincompatible mouse spleen cells have been cultured together in vitro. After 24 hours, cells have been treated with bromodeoxy uridine (BUDR) in the expectation that those lymphoid cells capable of reacting to the incompatible transplantation antigens will begin to divide, will incorporate BUDR and will then be incapable of reproducing further. Unstimulated lymphocytes should then be viable and capable of reconstituting an immunodepressed host. In vitro studies have confirmed those expectations and experiments will now be broadened to include reconstitution of appropriate recipients to determine whether a) GVH is avoided and b) whether reconstitution occurs.

Significance to Biomedical Research and the Program of the Institute: It is now possible to achieve complete restoration of immune function in individuals with selected immune deficiencies. The required levels of sophistication in the areas of immunologic evaluation, histocompatibility testing and bone marrow transplantation are available within the Immunology Branch. The experience to be gained from transplantation of immunocompetent cells in these individuals will improve our capacity to utilize this form of therapy in other patients with neoplastic disease. Long-term follow-up of these reconstituted individuals will enlarge our understanding of the effects of grafts on the immune system, and their influence on the evolution of neoplastic disease.

Proposed Course: More patients will be identified and evaluated and where indicated, grafts will be performed. In addition, appropriate experimental models in animals will be studied to assess means to broaden the approach of cellular reconstitution. In particular, the graft system in mice will be pursued to determine whether it is possible to avoid GVH in non-histocompatible combinations.

Honors and Awards: None

Publications: None

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Lymphocyte Stimulation: The Chemical Basis of Antigen Recognition and Gene Activation

Previous Serial Number: None

Principal Investigator: Steven A. Rosenberg, M.D., Ph.D.

Other Investigators: Ronald Levy, M.D.

Cooperating Units: None

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: 1) To chemically define the lymphocyte antigen receptors and the molecules involved in the early stages of lymphocyte activation in order to gain an understanding of the nature of the response of the sensitized lymphocyte to antigen. 2) To utilize these findings to develop sensitive assays for cellular immunity in man.

Methods Employed: Guinea pigs are immunized with protein antigens and serve as the source of lymphocytes for these studies. Antigen binding to lymphocytes is measured by new assays described below under "Major Findings". The chemical nature of antigen receptors has been studied by testing the effect of treating the lymphocyte with enzymes such as neuraminidase and papain and by using anti-guinea pig immunoglobulin antibodies. Membranes are isolated from lymphocytes by standard techniques of utilizing discontinuous sucrose gradients. Lymphocyte activation both by phytohemagglutinin and antigen has been measured using the incorporation of radioactive thymidine or radioactive amino acids. A double-label amino acid technique in the presence and absence of antigen has been used to identify immune specific proteins which are then fractionated using gel filtration chromatography and polyacrylamide gel electrophoresis.

Major Findings: An assay for detecting antigen receptors on lymphocytes

has been developed based on the ability of sensitized cells to absorb small quantities of antigen from solution. Changes in antigen concentration of pre- and post-absorptive solutions in the range of 1-10 nanograms/ml can be detected by measuring the ability of these antigens to stimulate the synthesis of DNA by sensitized lymphocytes. This assay enables the measurement of antigen binding by receptors at 1/100th the antigen concentration used in autoradiographic techniques. The binding of antigen by lymphocytes takes place in the presence of puromycin and mitomycin and thus is not dependent on continuing protein synthesis. Receptor function is not eliminated following removal of cell surface sialic acid by neuraminidase, thus implying the receptor binding site does not contain sialic acid. Receptor function is blocked by prior incubation of the cells with rabbit anti-guinea pig gamma globulin suggesting that the receptors may be immunoglobulins. Thus far, attempts to demonstrate receptor function on membrane fragments isolated from lymphocytes has not been successful.

Phytohemagglutinin activation of lymphocytes has been used as a model of lymphocyte activation by antigen. The use of sensitive double label techniques has enabled us to detect changes in protein synthesis of lymphocytes within 15 minutes after contact with phytohemagglutinin. These early proteins associated with cell activation are localized in the cytoplasm of the cells and do not seem to be present in the nucleus. Changes in protein synthesis by immune lymphocytes can be detected within four hours after contact with specific antigen. This stimulation exhibits carrier specificity, as has been demonstrated using dinitrophenyl derivatives of protein antigens, and thus may reflect the very early changes of the effector limb of cellular immunity. Studies in man are in progress to determine whether similar changes may be occurring in sensitized human lymphocytes when challenged with antigen.

Significance to Biomedical Research and the Program of the Institute: The mechanism of antigen recognition by immune lymphocytes represents an important gap in knwoledge of the activation of the immune system. Chemical characterization of lymphocyte receptors for antigen can lead to an understanding and possible manipulation of antigen activation of the immune response. The stimulation of lymphocytes by nonspecific mitogens and by specific antigens is an excellent model for the study of gene activation and control and thus parallels and be drawn to the regulation of gene function in differentiation, malignant transformation and other cellular phenomenon. By studying the early protein changes associated with lymphocyte activation, we hope to gain insight into the general phenomenon of gene activation in mammalian cells.

Current techniques for detecting cellular immunity to antigens in man take five to seven days (e.g. measurement of antigen stimulated DNA synthesis or mixed lymphocyte reactions). The development of a simple and rapid test for cellular immunity to soluble antigens in man, based on early changes in protein synthesis following lymphocyte activation, would be of use in studying the role of cellular immunity in cancer and in other human diseases.

Proposed Course of Project: Efforts to chemically characterize the lymphocyte receptors for antigen will continue by testing the effect of specific enzymes on receptor function and by attempting to separate active receptors from the intact cells. Fractionation of proteins synthesized early in the course of lymphocyte activation by both PHA and by specific antigen will continue. Attempts to duplicate these findings in man will be pursued in an effort to develop a sensitive in vitro assay for cellular immunity.

Honors and Awards: None

Publications: None

Serial No. NCI-5015 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Immune Response to Primary Cancers

Previous Serial Number: None

Principal Investigator: Steven A. Rosenberg, M.D., Ph.D.

Other Investigators: G. Nicholas Rogentine, Jr., M.D., Ronald B. Herberman, M.D. Albert B. Einstein, Jr., M.D., and John Wunderlich, M.D.

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: 1) To define the chemical nature of tumor membrane antigens, especially the role of sialic acid in the composition and masking of these antigens. 2) To study the cellular immune response to these antigens on primary human and animal tumors.

Methods Employed: Sialic acid is measured after hydrolysis in mild acid or after digestion with neuraminidase. Human lymphoid cells used in studies on the role of sialic acid in the HL-A antigen system are grown in continuous suspension culture using standard techniques. Synchronous cultures are induced using the double thymidine block technique. HL-A antigens of lymphoid cells before and after neuraminidase treatment have been quantitated using standard cytotoxic techniques and serial dilution of known antisera. Tumor specific antigens on RBL-5 mouse ascites tumors are measured by serial quantitative absorption of known antisera by tumor cells before and after neuraminidase treatment. Tumors were induced in guinea pigs using methylcholanthrene and are now beginning to appear (April, 1971). Cellular immune responses of of guinea pigs to their own primary cancers is studied using direct cellular cytotoxicity. An assay for cellular immunity in man has been developed based on the inhibition of leukocyte migration by specific antigen, and has been used to study the cellular immune response of humans to their own primary cancers.

Major Findings: The kinetics of neuraminidase action on cell surface sialic acids has been studied. 80% of the total human lymphoid cell sialic acid is on the cell surface membrane and is removed after 90 minutes treatment with neuraminidase (0.02 units/ml) at 37°C at pH 6.4. Under these conditions cell viability and growth is unaffected.

With Dr. Albert Einstein, it has been determined that the biosynthesis of the cell surface proteins containing sialic acid occurs within a 2-4 hour period in the G1 phase of the cell cycle just prior to the onset of mitosis. There are at least three major groups of membrane sialoproteins separable by gel filtration chromatography.

With Dr. G. Nicholas Rogentine, Jr., it has been determined that after the removal of all cell surface sialic acids no new HL-A antigens appear on human lymphoid cells and that the measured amount of the preexisting antigens is unchanged. Similar studies with Dr. Ronald Herberman on RBL-5 mouse ascites tumor cells have revealed a significant increase in the amount of the tumor specific antigens of this tumor after neuraminidase treatment.

An in vitro assay, based on the inhibition of leukocyte migration, to detect cellular immunity in man was developed in the previous year. This assay has been used to study the cellular immune response of humans to extracts of their own cancers. Thus far, ten patients with cancer have been studied and the existence of cellular immunity of patients to their own cancers have been demonstrated in six of these patients.

Significance to Biomedical Research and the Program of the Institute: Tumors grow in vivo despite the well demonstrated host immune responses developed against them. The explanation of this phenomenon is of fundamental importance to tumor immunology. Tumor antigens may be 'masked' and thus relatively ineffective in either the affector or effector limb of the host immune response. The role of cell membrane sialic acid in this possible 'masking' is being investigated in our studies. Human HL-A antigens do not appear to be masked though evidence for the masking of RBL-5 tumor antigens in mice has been presented above. These represent the first in vitro measurements of antigenicity before and after neuraminidase treatment. However, studies on primary tumors are the most relevant, and tumors are being induced in guinea pigs to perform these studies. An in vitro assay for detecting tumor antigens in humans is being evaluated (leukocyte migration assay) and may be of use in testing for tumor antigens, before and after modification, in man.

Proposed Course of Project: Studies on the biosynthesis of sialic acid containing proteins in cell membranes will focus on the fractionation and chemical characterization of these proteins.

Studies showing increased antigenicity to RBL-5 tumors after neuraminidase treatment will be continued in an effort to characterize this response and extend it to in vivo observations.

The major focus of these studies in the future will be on primary animal and human tumors. Autochthonous tumors induced in guinea pigs and mice will be tested for the role of sialic acid and other moieties in 'masking' tumor antigens. Similar in vitro studies of human cancers will be continued using the leukocyte migration assay or possibly a new double-label assay currently being developed.

Honors and Awards: None

Publications:

Rosenberg, S.A. and David, J.R.: Inhibition of leukocyte migration: An evaluation of this in vitro assay of delayed hypersensitivity in man to a soluble antigen. J. Immunol. 105: 1447, 1970.

Rosenberg, S.A. and David, J.R.: In vitro assay for inhibition of migration of human blood leukocytes. In Bloom, B. and Glade, P. (Eds.): Methods in Cellular Immunity. New York, N.Y., Academic Press, 1971.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cellular Immunity II

Previous Serial Number: None

Principal Investigator: John R. Wunderlich

Other Investigators: Eugene B. Rosenberg, M.D.

Contractor: Bionetics Research Laboratory

Contract # 70-2207
Money Allocated: $48,340

This progress report represents only a portion of work supported by contract #70-2207. Money allocated refers specifically to support for this project.

Cooperating Units: G.N. Rogentine, M.D., M. Blaese, M.D., D. Mann, M.D., N. Talal, M.D., R. Herberman, M.D., P. Levine, M.D. J. Potter, M.D., R. Malmgren, M.D., R. Yankee, M.D.

Man Years:

Total:	2 1/2
Professional:	1
Other:	1 1/2

Project Description:

Objectives: The project has been directed primarily at understanding cell-mediated immune cytotoxicity in humans. The long range goals are: 1) to identify human cells capable of destroying "foreign" cells by a cytotoxic, cell-mediated immune reaction; 2) to characterize factors which influence development of the cellular immune effector cells; 3) to characterize the mechanism of foreign cell destruction by effector cells; and 4) to define changes occurring in the cellular immune cytotoxic responses which accompany malignancies, immune deficiency diseases, organ transplantation, auto-immune states and iatrogenic immune suppression.

Methods Employed: Cell-mediated immune cytotoxicity is measured by

the release of chromium^{51} from labeled target cells incubated with immune lymphocytes. Target cells are prepared from peripheral blood, pathological fluid exudates, tissue culture lines, and biopsy specimens of lymph nodes and solid tumors. Solid tissue samples and cells adhering to tissue culture bottles are mechanically separated. Aggregated cells are allowed to settle. Red cells are then removed by ammonium chloride tris lysis. The resulting cell suspension is labeled with chromium^{51}. Normal and immune lymphocyte effector cells are prepared primarily from human peripheral blood. Heparinized peripheral blood is passed through nylon columns to remove polymorphonuclear cells and monocytes and then settled in plasmagel to reduce the red blood cell content. Our previous studies in a mouse model system have shown that cytotoxicity demonstrated with this assay is specific for cell-mediated reactions and independent of conventional complement. Subsequent studies have proved this system valuable for in vitro assessment of the cytotoxic effector stage of human cell-mediated immunity, measured independent of humoral immune activity.

Major Findings: Human Lymphocyte-Dependent Antibody. Early in 1970, we evaluated a patient with choriocarcinoma for cell-mediated immunity against her husband's peripheral blood lymphocytes. We postulated that she might have been immunized by her husband's transplantation antigens present on the tumor surface. No direct cell-mediated cytotoxicity was detected. Further tests were then run for the possible presence of "blocking antibody" in the patient's serum. Target cells from the husband were coated in vitro with the patient's serum and then incubated alone or with patient's lymphocytes. Coated target cells, incubated with patient's lymphocytes were extensively damaged - quite opposite of what would be expected from blocking antibodies.

The cell-dependent factor(s) in the patient's serum was an immunoglobulin by multiple criteria. On geon-pevikon block electrophoresis, activity migrated in the gammaglobulin region. It eluted from G-200 Sephadex in the 7S region. Activity was blocked, however, by anti-human immunoglobulin antisera, primarily anti-IgG; anti-IgM antisera did not block activity (samples kindly provided by Drs. A. Jackson, Fred Hymes and Roy Woods). Interference due to anti-IgG antisera was blocked by a purified IgG myeloma protein (kindly provided by Dr. William Terry). Specificity of the factor for target cells was demonstrated by coating target cells from seven separate normal human donors. Four of the donors' coated target cells were lysed in the presence of lymphocytes. Three were not. Target cells were not autotoxic because of their low concentration and inactivation of attacking cell function by ammonium chloride tris treatment. Factor specificity appeared to involve HL-A7. All reactive targets had HL-A7 (kindly assessed by Dr. G. Rogentine). Two non-reactive targets lacked HL-A7. The husband, but not patient possessed HL-A7. Activity was specific for the patient's serum. Blood samples from 10 normal donors lacked cell-dependent cytotoxic activity against lymphocytes from the patient's husband. Reactivity between coated target cells and attacking cells, however, was not species specific. Peripheral blood lymphocytes from 3 chimpanzees, 3 rhesus monkeys, a horse and a dog were cytotoxic for factor-coated target cells. Cell-mediated cytotoxicity fostered by the

humoral factor involved lymphocytes. Polymorphonuclear leukocytes and monocytes had been removed from the attacking cell preparations by nylon column filtration. Human lymphoid cells, adapted to long-term tissue culture, and red blood cells were not cytotoxic for factor-coated cells. The cytotoxic reaction is not akin to allogeneic inhibition in that the husband's peripheral blood lymphocytes were cytotoxic for autologous cells coated with lymphocyte dependent antibody. Nor is the cytotoxic reaction akin to non-specific mitogenic activation. PHA, one of the most potent mitogens, did not stimulate cytotoxicity, and the release of non-specific cell-free toxins (lymphotoxin) could not be detected.

The lymphocyte-dependent antibody characterized here may be a humoral IgX, an antigen-recognition immunoglobulin for cell-mediated immunity. Granted this, cellular immune effector cells would then consist not only of thymus dependent lymphocytes, synthesizing their own antibody recognition units, but also normal lymphocytes recruited by humoral IgX. A basic difference between cellular and humoral effector cells would be immunoglobulin attachment to and/or release from the effector cells. Judging by the absence of species specificity, the mode of lymphocyte-humoral IgX attachment is a primitive, relatively non-specific one. Rapid kinetics of target cell cytotoxicity by recruited lymphocytes showed that lag periods are not required for bolstering lysozyme activity or other normal lymphocyte functions; only the presence of recognition unit is required for toxicity.

In line with these speculations, peripheral blood lymphocytes from immune deficiency patients have been reacted with target cells coated with lymphocyte dependent antibody. In collaboration with Dr. Michael Blaese, these studies have involved patients with malignancies, Wiscot-Aldridge syndrome, intestinal lymphandrectasia, ectasia and hypogammaglobulinemia. Preliminary results show that patients anergic for cellular immunities by skin tests have lymphocytes which will destroy target cells coated with lymphocyte-dependent antibody. The patients appear unable to produce antigen-recognition antibody for cell-mediated immunity.

Concerning other areas of research work, patients with auto-immune disease are being evaluated for lymphocyte-dependent antibody (LDA) in collaboration with Drs. Dean Mann and Norman Talal. These patients have abnormally high levels of LDA activity against tissue culture lines and freshly explanted allogeneic cells.

Human cell-mediated cytotoxicity against autochthonous tumors is being evaluated, in collaboration with Drs. John Potter and Richard Malmgren. Primary efforts have been directed at developing mechanical methods for preparing viable target cells from solid tumors. On the basis of 18 solid tumor studies, direct cell-mediated cytotoxicity of presensitized lymphocytes for target cells can be detected, particularly in cases of lymph node and GI tract tumors. Human tumor work also included evaluation of 4 pairs of identical twins in cooperation with Drs. Herberman and Levine. One of each twin pair had leukemia. Cell-mediated cytotoxicity studies revealed activity

of both family members and unrelated donors against cells from the leukemic twins. Activity was not detected against the normal twins.

Significance to Biomedical Research and the Program of the Institute: Techniques now exist for monitoring cytotoxic human cellular immune activity in vitro. Further understanding of this mechanism will be particularly valuable in the fields of cancer transplantation, auto-immune disease, and in infectious diseases where cell-mediated immunity is felt to play a major role in host defense mechanisms. Identification of human lymphocyte-dependent antibody is important both from basic and practical standpoints. Use of this antibody has permitted partial dissection of a mechanism for cell-mediated cytotoxicity, characterization of a recognition factor (antibody) for cell-mediated cytotoxicity, and investigations of lymphocytes which can be recruited as effector cells for cellular immunity. From a practical standpoint, lymphocyte-dependent antibody must be considered apart from complement-dependent antibody in the cytotoxic humoral immune response. Lymphocyte-dependent antibody also is clearly independent of humoral antibody which blocks target cell destruction by cell-mediated immunity.

Proposed Course of Project: Further studies of lymphocyte dependent antibody will be directed at the mechanism of target cell destruction and the role played by recruited, non-sensitized lymphocytes in this process.

Work will continue on identifying the most effective means of measuring cell-mediated cytotoxicity against autochthonous tumors in vitro. More effort is needed for developing cell preparation schemes which preserve both immune and target cell integrity.

Honors and Awards: None

Publications:

Wunderlich, J.R., Rosenberg, E., and Connolly, J. (1971). Human Lymphocyte-Dependent Cytotoxic Antibody and Mechanisms of Target Cell Destruction in vitro. In "Proceedings First International Congress of Immunology". Academic Press, New York, in press.

ANNUAL REPORT OF THE METABOLISM BRANCH
SUMMARY OF SIGNIFICANT ACTIVITIES
NATIONAL CANCER INSTITUTE

July 1, 1970 through June 30, 1971

The research of the Metabolism Branch is directed toward two major goals. The first is to define the physiological and biochemical abnormalities produced by a tumor on the host. Both patients with neoplastic disease as well as those with non-neoplastic disorders that permit the development of techniques to study metabolic derangements are being investigated. The second major goal is to define the host factors that result in a high incidence of neoplastic disease. The patients studied in this area are those with immunological deficiency diseases such as the Wiskott-Aldrich syndrome, ataxia telangiectasia and intestinal lymphangiectasia where the immunological surveillance mechanisms that are important for the elimination of neoplastic disease do not function normally.

Studies of the Physiology and Pathophysiology of the Immune Response

The factors involved in the ontological development of the immune response were studied in neonatal rats. It was demonstrated that newborn rats cannot produce antibodies to a number of antigens. The ability to respond to different antigens develops in a distinctive stepwise fashion. It was demonstrated that limiting factor in immune responsiveness in the neonatal rats is macrophage immaturity rather than a lack of specific antibody-forming precursor cells. Ordinarily, unresponsive neonatal animals can produce antibody to a variety of antigens if these antigens are given along with mature adult macrophages. Maturation of the macrophages of the neonate can be promoted by several materials so that adult cells need not be transplanted to allow neonatal antibody formation. Brucella vaccine, pertussis vaccine, alum, endotoxin and poly IC function as macrophage-activating agents and also as immunologic adjuvants. The efferent linked bacterial killing function of macrophages are also activated in the neonate by these agents. Listeria monocytogenes infection is usually lethal to newborn rats, but macrophage activation results in protection and survival from the infection. The relative ease with which neonatal animals can be made immunologically tolerant can also be prevented by providing neonates with mature macrophages or by macrophage-activating substances given to the neonates. Thus, manipulation of the state of activation of macrophages permits another level of control of specific immune responses. These studies may be considerably significant in developing immunity to vertically transmitted virally induced tumors.

Factors controlling immune responses in individual immunoglobulin classes, particularly the IgA class, was studied using the C'1a fixation and transfer test. It was found that intravenous immunization with the soluble protein antigen, bovine serum albumin, elicited little or no antibody response, whereas a larger protein more readily taken up by the reticuloendothelial system, such as keyhole limpet hemocyanin, did result in an IgA antibody response. Similarly, incorporation of bovine serum albumin in complete Freund's adjuvant or chemical modification of bovine serum albumin (diazotization with arsanilic acid) resulted in IgA antibody responses; each of

these procedures are again associated with greater reticuloendothelial system uptake. Finally, antibody responses to intravenous arsanilic acid on a BSA carrier when given intravenously leads to a relatively small IgA response, whereas the same antigen incorporated into complete Freund's adjuvant leads to exceedingly large IgA responses. These findings indicate that reticuloendothelial system participation is a necessary component in the initiation of IgA antibody responses.

Previous studies in this laboratory have established that neonatal thymectomy of rabbit is associated with diminished antibody responses to intravenously administered arsanilic acid on a bovine serum albumin carrier. In current studies it was shown that the same antigen, arsanilic acid on a keyhole limpet hemocyanin carrier, did evoke an IgA antibody response. This indicates that thymus-dependent helper cells are necessary for the initiation of IgA responses and may constitute one component of the reticuloendothelial participation mentioned above.

In recent studies, a system involving the transcellular transport of immunoglobulins has been defined. This system involves the specific uptake and transport of IgG molecules across the gastrointestinal track of neonatal rats. All four subclasses of human IgG, rat IgG and rabbit IgG are involved in this process. None of the four other classes of immunoglobulins or other serum proteins are involved in this specific transport system. Both the uptake and transport are saturable processes in that the rate of transport of unlabeled IgG can be reduced 10-fold when excess unlabeled IgG is administered simultaneously. We have shown that the IgG in intestinal wall migrates on sucrose gradients as a very high molecular weight complex. Using subcellular fractionation studies, it has been shown that the IgG is bound to a cell membrane receptor that is specific for the IgG Fc piece. The specific transport of IgG thus involves the binding of IgG to a limited number of cell-surface membrane receptors. Following binding, the IgG is transported to the villus lymphatic channels in pinocytotic vessicles. The possibility that a similar process of membrane binding to explain the specific transfer of IgG across the human placenta is being investigated.

The studies of the physiology and pathophysiological factors controlling the synthesis, transport and catabolism of immunoglobulins were extended with studies of the metabolism of IgE, the reagenic antibody, in various disorders. A new double antibody assay for the quantitation for IgE concentrations was developed for use in these studies. Normal individuals had a geometric mean IgE serum concentration of 79 ng/ml. The IgE synthetic rate was 30 ng/kg/day and the fractional catabolic rate was 89% of the intravenous pool per day. The IgE level was determined in the serum of 20 patients with ataxia telangiectasia. In 12 of these patients, there was no detectable IgE or IgA. In three additional patients, there was a marked reduction in the IgE level but normal IgA levels; and in 2 patients, both IgE and IgA levels were normal. IgE levels were also undetectable in 5 blood bank donors who did not synthesize IgA. All of these patients with low IgE levels had a markedly reduced rate of IgE synthesis to less than 1% of normal, whereas the IgE fractional catabolic rate was normal. Low rates of IgE synthesis were also noted in patients with idiopathic agammaglobulinemia and with agammaglobulinemia associated with thymomas.

Markedly increased serum levels of IgE and increased IgE synthetic rates were noted in patients with the Wiskott-Aldrich syndrome, patients with schistosomiasis, and patients with Hodgkin's disease. One patient who produced an IgE myeloma protein had a markedly prolonged IgE survival in addition to the increased synthetic rates for IgE. IgE is normally fixed to a variety of tissues including skin, basophils, and lungs. It is possible that similar binding is involved in its catabolism. Patients with IgE myeloma may have saturated the specific IgE catabolic binding sites and have a reduced fractional catabolic rate as a consequence.

Studies on the catabolism of low molecular weight serum proteins including Bence Jones proteins and lysozyme by the kidney have been continued. In these studies, the kidney has been shown to play a major role in the catabolism of L-chains, Bence Jones proteins, lysozyme and ribonuclease. Patients with proximal renal tubular disease have normal synthetic rates and glomerular clearance of these proteins, but because of a failure of tubular uptake and catabolism they excrete from 50-100-fold increased levels of these proteins in the urine. Patients with uremia and nephron loss have lost the normal site of catabolism and excretion of these proteins. Consequently, the serum levels of such proteins as L-chains and lysozyme are increased 10-fold. This increase in the serum concentration of biologically active small molecules including enzymes and hormones may be of significance in the pathogenesis of the uremic syndrome.

In association with Dr. J. Wunderlich and E. Rosenberg, studies of in vitro lymphocyte function have been carried out in 18 anergic patients with immune deficiency or cancer. Clinical evaluation of cellular immune competence was compared with in vitro lymphocyte transformation responses to nonspecific mitogens, specific antigens, and allogeneic cells in mixed leukocyte culture. In addition, the ability of lymphocytes from these patients to perform cell-mediated cytotoxic reactions with target cells coated with lymphocyte-dependent antibody (LDA) were determined. Patients with the Wiskott-Aldrich syndrome have marked clinical anergy with inability to manifest delayed type hypersensitivity skin tests to agents such as PPD, mumps, Candida, SK-SD, and diphtheria and tetanus toxoids. They were unresponsive following challenge with DNCB, and also showed delayed rejection of skin homografts. The in vitro transformation of lymphocytes from these patients showed normal responses to nonspecific mitogens with marked impairment in responses to specific antigens and allogeneic cells. Cytotoxicity reactions by lymphocytes from these patients were normal to LDA-coated target cells. Patients with advanced cancer or intestinal lymphangiectasia also had marked clinical anergy, and in addition, generally had severely impaired in vitro lymphocyte transformation responses to all classes of stimulants. However, these patients also had normal lymphocyte responses in LDA cytotoxicity reactions.

Studies of chylous effusion lymphocytes in patients with intestinal lymphangiectasia showed more normal responses to mitogens, but impaired responses in LDA cytotoxicity. Thus, these studies have identified at least three separable in vitro lymphocyte functions (transformation responses to nonspecific mitogens, transformation to antigens and allogeneic cells, and LDA cytotoxicity reactions) and suggests that recirculating long-lived lymphocytes

are responsible for blastogenic responses; while short-lived, nonrecirculating lymphocytes participate in LDA-cytotoxicity reactions.

Studies on the nature of the immunologic defect in ataxia-telangiectasia were continued. Ataxia-telangiectasia is an autosomal recessive disease characterized by the presence of cerebellar ataxia, oculocutaneous telangiectasia, increased frequency of sinopulmonary infections, and reticuloendothelial neoplasms. In previous studies, it was established that patients with this disease have a 70-75% incidence of IgA immunoglobulin deficiency. Using the C'1a fixation and transfer test, the antibody response in individual immunoglobulin classes to group substance was studied. Normal persons have responses in all three major immunoglobulin classes, whereas ataxia patients who lack IgA have responses only in the IgG and IgM classes. Surprisingly, those patients with ataxia telangiectasia with normal or even elevated IgA levels also had no response in the IgA immunoglobulin class. This indicates that there is a functional deficiency in IgA responses, even in patients that synthesize IgA.

In association with Dr. Z. Myron Falchuk, continued studies on the factors controlling local gastrointestinal responses were conducted. A new method for the measurement of local gastrointestinal immunoglobulin synthesis (IgA and IgM) were developed. This method depends on the measurement of ^{14}C-leucine incorporation into immunoglobulins by jejunal biopsy in short-term culture. The indicator system consists of anti-immunoglobulins affixed to the solid phase immuno-absorbant, bromacetyl cellulose. Studies were carried out demonstrating the specificity and linearity of this system; in addition, normal values for local immunoglobulin production in normal persons were established. Using this method, it was found that patients with gluten-induced enteropathy had greatly elevated incorporation values, whereas patients with hypogammaglobulinemia had very low values. This system is more quantitative than immunofluorescent methods and should be of great value in the assessment of immunological factors in gastrointestinal disease.

Patients with gluten-induced enteropathy underwent continued study for evaluation of immunological factors in the pathogenesis of this disease. Previous results showing increased local immunoglobulin synthesis soon after gluten challenge were confirmed using the solid-phase immuno-absorbant techniques described above. In addition, local production of specific antigluten antibody was demonstrated in patients following a gluten-challenge, but not in prechallenge biopsy specimens. This was done by specific binding of incoporated ^{14}C-leucine counts to Sepharose columns to which had been attached the gluten antigen. It was found that a very considerable part (30-70%) of the total increase in immunoglobulin production following gluten challenge could be accounted for by the antigluten antibodies. These studies further implicate immunologic processes in the pathogenesis of gluten-induced enteropathy.

Porphyrin Metabolism

A total of six hepatomas, a fibrosarcoma and the Walker carcinosarcoma have now been studied for induction of δ-aminolevulinic acid synthetase (ALAS). The enzyme could not be induced by allylisopropylacetamide (AIA) in any of

the tumors, but tyrosine aminotransferase was induced by ALA in all tumors, usually to a greater extent than in normal liver. The presence of a tumor markedly impairs induction of ALAS in the host's liver, but does not impair induction of hepatic tyrosine aminotransferase in the host's liver. Studies of hemopexin and methemalbumin levels have ruled out the possibility that heme released from red cell or necrotic tumor cells is the cause of the effect on the host's liver. Excessive lactate production by tumors is not producing a "glucose effect" on the host liver, and an inhibitor of hepatic ALAS could not be demonstrated in tumors. Preliminary data suggest a nondialyzable substance in tumors which blocks the induction of hepatic ALAS.

Studies of skin fibroblasts of patients with acute intermittent porphyria indicate a decreased rate of conversion of porphobilinogen to porphyrins as well as some increase of ALAS. This indicates that the defect present in liver in this disease may also be present in milder degree in other cells. The respiration of porphyric fibroblasts has been studied in collaboration with Dr. Eugene Weinbach who has demonstrated a decreased rotenone inhibition of respiration in these cells. This indicates defective NADH dehydrogenase. The cells respond normally to other respiratory stimulants and inhibitors.

The effect of glycerol on porphyrin precursor excretion has been studied in patients with acute intermittent porphyria. Some respond better to glycerol than glucose and some respond about the same to both agents.

Amino Acid Transport

The mechanism of cyclic AMP stimulation of amino acid transport in rat kidney cortex was studied. The synthesis of a mediator appears to be involved as indicated by: (1) Occurrence of the effect requires at least two hours of exposure to dibutyryl cyclic AMP (DBC). After the period of exposure to DBC, tissue transferred to incubation medium without DBC shows undiminished stimulation of amino acid transport. (2) Kinetics studies show an increase in transport V_{max}, but no change in K_m. (3) The addition of puromycin and cycloheximide abolishes the DBC stimulation. (4) The stimulation of amino acid transport involves a process dependent on Na-K ATPase. The deletion of sodium or the addition of ouabain blocks the DBC effect.

The above findings suggested that DBC stimulates the synthesis of a transport mediator dependent on Na-K ATPase.

Studies of the Na-K ATPase isolated from kidney cortex showed: (1) Cyclic AMP has no direct effect on the enzyme. (2) The amount of Na-K ATPase per mg protein was unchanged in DBC-treated tissue. (3) The enzymes from DBC-treated and control tissues showed identical affinities for ATP. However, the DBC-treated enzyme showed increased affinity for phosphate at Na = 20 mM and K = 5 mM, concentrations of sodium and potassium which approximate the physiologic exposure of the enzyme to these ligands *in situ*. These studies suggest that cyclic AMP induces the production of a modified Na-K ATPase with a greater affinity for phosphate but unaltered affinity for ATP, thus allowing for accumulation of a high-energy phosphoenzyme complex which participates in active amino acid transport.

The effect of cyclic AMP on amino acid uptake and collagen synthesis in bone was studied using fetal rat calvaria. Using L-proline-U-^{14}C it was shown that DBC increases the uptake of proline as well as the incorporation of proline into protein and collagen. The DBC effect is sensitive to medium phosphate concentration. Stimulation of collagen synthesis is maximal at a medium phosphate concentration of 1.2 mM. Higher phosphate concentrations decrease the DBC effect. In fact, at a phosphate concentration of 2.4 mM, DBC produces significant inhibition of collagen synthesis.

The phosphate modification of the DBC effect appears to be mediated through phosphate effects on transport. DBC stimulation of AIB transport was abolished at phosphate concentrations of 2.4 mM. The phosphate effect on transport was not due to phosphate interference with DBC entry. Tissue exposed to DBC in buffer with phosphate concentrations of 1.2 mM showed undiminished stimulation of AIB transport.

Although phosphate modified the DBC effect on both amino acid transport and collagen synthesis, changes in calcium concentration had no observable effects. Calcium concentrations between 1.2 and 5 mM produced no change in DBC stimulation.

Cyclic AMP regulation of collagen synthesis is modified by phosphate. The finding suggests that cyclic AMP and phosphate play a physiologic role in collagen metabolism.

Calcium Metabolism

We have investigated the effects of diphosphonates in various diseases in man. Diphosphonates, a family of compounds with standard similarities to pyrophosphate inhibits both calcification and bone resorption. Unlike pyrophosphate, diphosphonates are resistant to enzymatic hydrolysis by pyrophosphate. In patients with myositis ossificans progressiva and calcinosis universalis, diphosphonates produce marked clinical improvement. ^{47}Ca kinetics and balance studies show decreased bone calcium turnovers and decreased hydroxyproline excretion in patients on the drug. Calcium balance, however, is unaffected. Thus, the pharmacologic effect occurs without demineralization of normal skeletal bone.

Bilirubin Metabolism

Studies of radiobilirubin kinetics have been performed in additional normal volunteers and patients with a variety of disease states. From these studies, the rates of hepatic bilirubin clearance and bilirubin production were defined for a normal population. Since these are the two principal factors which determine the plasma concentration of unconjugated bilirubin, it was possible to derive, on theoretical grounds, a distribution function for bilirubin concentration in a normal population, and to accurately define an upper limit of normal.

Studies of bilirubin kinetics were performed before and during fasting in humans and rats in order to study the mechanism for the recently described

phenomenon of "fasting hyperbilirubinemia." The studies demonstrated that a significant reduction in hepatic bilirubin clearance after 48 or more hours of fasting accounted for this phenomenon.

The effect of glutethimide, a hypnotic structurally related to phenobarbital, has been examined in a group of patients with Gilbert's syndrome and normal volunteers. Glutethimide, given as 500 mgm p.o. q.h.s., produced a significant reduction in the plasma concentration of unconjugated bilirubin in all subjects. In Gilbert's syndrome, initial values of 1.7 $\pm$ 0.5 mgm % fell to normal within one week, and averaged 30% of baseline after 2 weeks. Similar reductions occurred in the normal volunteers. Studies with labeled bilirubin, both before and during therapy, indicated that the fall in plasma bilirubin concentration resulted from both an increase in hepatic bilirubin clearance and a decrease in bilirubin production. An increase in bilirubin clearance and a reduced plasma bilirubin concentration, but no fall in bilirubin production, were observed in phenobarbital treated patients.

Following intravenous administration of isotopically labeled δ-aminolevulinic acid (δ-ALA), labeled unconjugated bilirubin circulates in the plasma. This labeled bilirubin is formed almost exclusively in the liver. Mathematical techniques have been developed by which the amount of isotopically labeled precursor incorporated into bilirubin can be calculated. Studies have been done in two normal individuals, two patients with red cell aplasia, and three patients with acute intermittent porphyria. In the porphyrics, application of the labeled precursor-product relationship to the labeling of both bilirubin and urinary porphobilinogen following administration of labeled δ-ALA permitted the first *in vivo* determinations of hepatic bilirubin production.

Serial No. NCI-4002(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Physiology and Pathophysiology of Plasma Protein Metabolism

Previous Serial Number: SAME

Principal Investigator: Thomas A. Waldmann, M.D.

Other Investigators: Warren Strober, M.D., R. Michael Blaese, M.D. and William Terry, M.D.

Cooperating Units: Immunology Branch, NCI

Man Years

Total: 3
Professional: 2
Others: 1

Project Description:

Objectives: The objectives of the study were to determine the normal rate of synthesis, transport and catabolism of the plasma proteins, especially the immunoglobulins. To determine the physiological factors controlling these metabolic parameters and to determine the pathophysiology of disorders of serum protein metabolism in patients with neoplastic disease and in patients with hereditary disorders of the immunoglobulin metabolism.

Methods Employed: The rates of synthesis, catabolism and distribution of the serum proteins were studied using radioiodine labeled purified proteins including albumin, the five major groups of immunoglobulins, as well as immunoglobulin fragments. These proteins were isolated from the serum and urine using physical chemical procedures including DEAE cellulose and Sephadex chromatography and zone acrylamide gel electrophoresis. The iodinated and purified proteins were either labeled in vitro with ^{131}I, ^{125}I or ^{51}Cr. The curves of radioactivity in the urine, serum, stool nasal, and salivary secretions were used with mathematical multicompartmental models to determine the rates of protein synthesis, catabolism and distribution into the extravascular compartments and external secretions. In addition, sucrose gradient ultracentrifugation as well as the passage of proteins through millipore membranes were used in new techniques for the demonstrations of IgG receptors on the surface of cells.

Major Findings: The transport of IgG across the intestinal mucosa of neonatal rats were used as a model for the study of transcellular protein transport. The mechanism of intestinal uptake and transcellular transport of plasma proteins has been studied in 14-day-old rats using intraduodenally administered radioiodinated proteins. Appreciable quantities of rat IgG, mouse IgG, and all four subclasses of human IgG were taken up by the intestinal wall (19-52% of the administered dose at 4 hours) and transported to the animal (10-36% of the administered dose at 4 hours). In contrast there was little or no uptake or transport of human IgM, IgA, IgD, IgE, albumin, transferrin, and ceruloplasmin. Both the uptake and transport of labeled IgG were significantly inhibited by unlabeled IgG. An appreciable proportion of the label of IgG in intestinal wall homogenates but not in plasma or intestinal washings migrated in a sucrose ultracentrifugation gradient much more rapidly than did the administered 7S molecules. This pattern was not observed with other proteins studied. This apparent binding of labeled IgG was also markedly inhibited by unlabeled IgG. Using subcellular fractionation studies it was shown that the IgG is bound to a cell membrane receptor that is specific for the IgG Fc piece. It is concluded that the neonatal rat both intestinal uptake and transport of IgG are specific saturable processes; that this intestinal transport is associated with binding of IgG molecules to cell membrane receptors. Following binding to the receptors the IgG molecules are transported in pinocytotic vessicles to the villus lymphatics. The possibility that a similar process of membrane binding exists to explain a specific transfer of IgG across the human placenta is being investigated. The studies of the physiology and pathophysiological factors controlling the synthesis, transport and catabolism of immunoglobulins were extended with the studies of IgE, the reagenic antibody in various disorders. A new double antibody assay for the quantitation of IgE concentrations was developed for the use in these studies. Normal individuals had a geometric mean IgE serum concentration of 79 ng/ml. The IgE synthetic rate was 30 ng/kg/day and the fractional catabolic rate was 89% of the intravenous pool/day. The IgE level was determined in the serum of 20 patients with ataxia telangiectasia. In 12 of these patients there was no detectable IgE or IgA. In 3 additional ataxia telangiectasia patients there was a marked reduction in the IgE level but normal IgA levels were normal. IgE levels were also undetectable in 5 blood bank donors who do not synthesize IgA. All these patients with low IgE levels had a profound defect in IgE synthesis to less than 1% of normal, whereas IgE fractional catabolic rates were normal. Low rates of IgE synthesis were also noted in patients with idiopathic agammaglobulinemia and with agammaglobulinemia associated with thymomas. Markedly increased serum levels of IgE and increased IgE synthetic rates were noted in patients with schistosomiasis and patients with Hodgkin's disease. One patient who produced an IgE myeloma protein had a markedly prolonged IgE survival in addition to the increased synthetic rates for IgE. IgE is normally fixed to a variety of tissues including skin, basophils and lungs. It is possible that similar binding is involved in its catabolism. Patients with IgE myeloma may have saturated the specific IgE catabolic binding sites and have a reduced fractional catabolic rate as a consequence.

Studies on the catabolism of low molecular weight serum proteins including Bence-Jones and lysozyme by the kidney have been continued. In these studies the kidney has been shown to play a major role in the catabolism of L-chains, Bence-Jones proteins, lysozyme, and ribonuclease. Patients with proximal renal tubular disease have a normal synthetic rate and glomerular clearance of these proteins but because of a failure of tubular uptake and catabolism they excrete from 50 to 100-fold increased levels of these proteins in the urine. Patients with uremia and nephron loss have lost the normal site of catabolism and excretion of these proteins and consequently the serum levels of such proteins of L-chains and lysozyme increase 10-fold. This increase in the serum concentration of biologically active small molecules including enzymes and hormones may be of significance in the pathogenesis of the uremic syndrome.

A new syndrome with hypogammaglobulinemia associated with a short immunoglobulin survival due to an abnormal protein-protein interaction was defined in a patient with Sjögren's syndrome. This patient had a mixed IgM-IgG cyroglobulinemia, a high serum level of monoclonal IgG reactive IgM, a slightly elevated concentration of IgG_3 and a markedly reduced concentration of the other IgG subclasses. In this case the monoclonal IgM form complexes with IgG_1, IgG_2 and IgG_4 subclasses over a range of temperatures obtainable *in vivo*. The rates of synthesis of these subclasses were significantly reduced, presumably because the IgG reactive IgG-IgM molecules interacted with the cell surface receptors of cells that are precursors of immunoglobulin producing cells. The survival of these subclasses were also markedly shortened in the patients presumably due to the formation *in vivo* of IgM-IgG complexes. IgG_3 that did not form complexes with the monoclonal IgM had a normal survival. These studies are the first indicating a short survival of some IgG subclasses in a patient with a normal survival of another subclass.

Significance to Bio-medical Research: The present studies extend our understanding of the normal metabolism and physiological control mechanisms that affect immunoglobulin synthesis, transport, and catabolism. They indicate that part of this process may involve the binding of immunoglobulins to cell-surface receptors. They confirm that disorders of serum protein levels may occur due to a variety of pathophysiological mechanisms including decreased synthesis, accelerated catabolism or excessive loss of these proteins. High levels of Bence-Jones proteins and lysozyme in the urine may occur not only due to increased synthesis associated with neoplastic disease, but also secondary to renal tubular defects, a phenomenon of significance in the diagnosis of certain neoplastic disorders. Many of the disorders of immunoglobulin metabolism were discovered in diseases associated with high incidence of malignancy.

Proposed Course: Continued studies directed toward understanding the pathways and controlling mechanisms of the synthesis, transport and catabolism of the serum proteins.

Honors and Awards

Superior Service Award

Publications

Butler, W. T., Rossen, R. D. and Waldmann, T. A.: Local synthesis of immunoglobulin G in the upper respiratory tract. In Peeters, H. (Ed.): Protides of the Biological Fluids. Elsevier Press, 1969, pp. 331-335.

Waldmann, T. A., Strober, W. and Blaese, R. M.: Turnover and metabolism of immunoglobulins. In Merler, E. (Ed.): Biological Aspects and Clinical Uses of Immunoglobulins. Washington, D. C., National Academy of Sciences, 1970, pp. 33-51.

Strober, W., Blaese, R. M. and Waldmann, T. A.: The origin of salivary IgA. J. Lab. Clin. Med. 75: 856-862, 1970.

Morell, A., Terry, W. D. and Waldmann, T. A.: Metabolic properties of IgG subclasses in man. J. Clin. Invest. 49: 673-680, 1970.

Levy, A. L. and Waldmann, T. A.: The effect of hydrocortisone on immunoglobulin metabolism. J. Clin. Invest. 49: 1679-1684, 1970.

Strober, W., Blaese, R. M. and Waldmann, T. A.: Abnormalities of immunoglobulin metabolism. In Rothschild, M. A. and Waldmann, T. A. (Eds.): Plasma Protein Metabolism - Regulation of Synthesis, Distribution, and Degradation. New York, Academic Press, 1970, pp. 287-304.

Waldmann, T. A., Blaese, R. M. and Strober, W.: Physiological factors controlling immunoglobulin metabolism. In Rothschild, M. A. and Waldmann, T. A. (Eds.): Plasma Protein Metabolism - Regulation of Synthesis, Distribution, and Degradation. New York, Academic Press, 1970, pp. 269-285.

Rothschild, M. A. and Waldmann, T. A.: Plasma Protein Metabolism - Regulation of Synthesis, Distribution, and Degradation. New York, Academic Press, 1970, 582 pages.

Shimpkin, P. M., Waldmann, T. A. and Krugman, R. L.: Intestinal lymphangiectasia. Amer. J. Roent. 110: 827-841, 1970.

Miller, W. V., Holland, P. V., Sugarbaker, E., Strober, W., and Waldmann, T. A.: Anaphylactic reactions to IgA: A difficult transfusion problem. Amer. J. Clin. Path. 54: 618-621, 1970.

Mogielnicki, R. P., Strober, W. and Waldmann, T. A.: Renal handling of low molecular weight proteins. I. L-chain metabolism in experimental renal disease. J. Clin. Invest. 50: 901-909, 1971.

Waldmann, T. A., Johnson, J. and Talal, N.: Hypogammaglobulinemia associated with accelerated catabolism of IgG secondary to its interaction with an autoreactive monoclonal IgM. J. Clin. Invest. 50: 951-959, 1971.

Butler, W. T., Waldmann, T. A., Rossen, R. D., Douglas, R. G., Jr., and Couch, R. B.: Changes in IgA and IgG concentrations in nasal secretions prior to the appearance of antibody during viral respiratory infection in man. J. Immunol. 105: 584-591, 1970.

Langer, T., Strober, W. and Levy, R. I.: The metabolism of plasma lipoproteins. In Rothschild, M. A. and Waldmann, T. A. (Ed.): Plasma Protein Metabolism - Regulation of Synthesis, Distribution, and Degradation. New York, Academic Press, 1970, pp. 484-500.

Wochner, R. D., Drews, G., Strober, W., and Waldmann, T. A.: IgG metabolism in myotonic dystrophy. Proceedings of the Second International Symposium on the Biochemistry of Muscle Disorders, Montreal. In press.

Waldmann, T. A.: Intestinal lymphangiectasia. In Bergsma, D. (Ed.): Birth Defects Compendium. The National Foundation Press. In press.

McFarlin, D. E., Strober, W., Barlow, M., and Waldmann, T. A.: The immunological deficiency state in ataxia-telangiectasia. In Research Publications of the Association for Research in Nervous and Mental Disease. In press.

Gill, J., Waldmann, T. A. and Bartter, R.: Abnormal albumin metabolism in idiopathic edema. J. Clin. Invest. In press.

Ogawa, M., McIntyre, O. R., Ishizaka, K., Ishizaka, T., Terry, W. D., and Waldmann, T. A.: Biological properties of E-myeloma proteins. Amer. J. Med. In press.

Waldmann, T. A., Strober, W. and Mogielnicki, R. P.: The proteinuria of cystinosis: Its pattern and pathogenesis. Cystinosis. In press.

Blaese, R. M., Strober, W., Levy, A. L., and Waldmann, T. A.: Hypercatabolism of IgG, IgA, IgM, and albumin in the WAS: A unique disorder of serum protein metabolism. J. Clin. Invest. In press.

Serial No. NCI-4003(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria

Previous Serial Number: SAME

Principal Investigator: Donald P. Tschudy, M.D.

Other Investigators: Herbert L. Bonkowsky, M.D. and Annie Collins

Cooperating Units: Virus and Disease Modification Section, Viral Leukemia and Lymphoma Branch. Laboratory of Parasitic Diseases, NIAID

Man Years

Total: 4
Professional: 2
Others: 2

Project Description:

Objectives: To study the mechanisms controlling heme biosynthesis and biochemical and clinical aspects of the porphyrias. To apply the knowledge gained from the above objectives to the study of tumors and tumor-host relationships.

Methods Employed: (A) Quantitative enzyme determinations. (B) Chemical determination of porphyrins and porphyrin precursors. (C) Use of tissue homogenates, slices and mitochondria in studying metabolic pathways.

Major Findings: A mathematical model has been developed which relates the values of parameters of enzyme activity to pool sizes of substrates in an irreversible metabolic pathway at equilibrium. The model shows that the concentration of a substrate at any step in an irreversible pathway is a function of 5 variables: a) the precursor substrate concentration, b) the K_M of the enzyme which forms the substrate, c) the V_m of the enzyme which forms the substrate, d) the K_M of the enzyme which utilizes the substrate and e) the V_m of the enzyme which utilizes the substrate. It also defines the conditions under which an irreversible pathway can achieve a steady state. This model can be applied to the effects of inborn errors of metabolism and chemical agents on metabolic pathways such as those of heme, cholesterol, purine and pyrimidine biosynthesis.

A total of 6 hepatomas, a fibrosarcoma and the Walker carcinosarcoma have now been studied for induction of δ-aminolevulinic acid synthetase (ALAS). The enzyme could not be induced by allylisopropylacetamide (AIA) in any of the tumors, but tyrosine aminotransferase was induced by AIA in all tumors, usually to a greater extent than in normal liver. The presence of a tumor markedly impairs induction of ALAS in the host's liver, but does not impair induction of hepatic tyrosine aminotransferase in the host's liver. Studies of hemopexin and methemalbumin levels have ruled out the possibility that heme released from red cells or necrotic tumor cells is the cause of the effect on the host's liver. Excessive lactate production by tumors is not producing a "glucose effect" on the host liver and an inhibitor of hepatic ALAS could not be demonstrated in tumors. Preliminary data suggest a non-dialyzable substance in tumors which blocks the induction of hepatic ALAS.

Studies of skin fibroblasts of patients with acute intermittent porphyria indicate a decreased rate of conversion of porphobilinogen to porphyrins as well as some increase of ALAS. This indicates that the defect present in liver in this disease may also be present in milder degree in other cells. The respiration of porphyric fibroblasts has been studied in collaboration with Dr. Eugene Weinbach who has demonstrated a decreased rotenone inhibition of respiration in these cells. This indicates defective NADH dehydrogenase. The cells respond normally to other respiratory stimulants and inhibitors.

The effect of glycerol on porphyrin precursor excretion has been studied in patients with acute intermittent porphyria. Some respond better to glycerol than glucose, some respond not as well to glycerol as to glucose and some respond about the same to both agents.

Significance to Bio-medical Research: Mechanisms of the control of metabolic pathways and the synthesis of enzymes in normal and diseased tissues are being elucidated. New abnormalities of control mechanisms in tumors have been demonstrated and will be studied further.

Proposed Course: (A) To continue studies of the factors controlling heme and heme-enzyme synthesis in normal and pathologic tissues. (B) To continue studies of biochemical abnormalities of acute porphyria in man and experimental animals. (C) To study control mechanisms of heme synthesis in tumors and the livers of tumor-bearing hosts.

Honors and Awards

Superior Service Award

Publications

Berk, P.D., Tschudy, D. P., Shepley, L. A., Waggoner, J. G., and Berlin, N. I.: Hematologic and biochemical studies in a case of lead poisoning. Amer. J. Med. 48: 137-144, 1970.

Stein, J. A., Tschudy, D. P., Corcoran, P. L., and Collins, A.: δ-aminolevulinic acid synthetase. III. Synergistic effect of chelated iron on induction. J. Biol. Chem. 245: 2213-2218, 1970.

Stein, J. A., Bloomer, J. R., Berk, P. D., Corcoran, P. L., and Tschudy, D. P.: The kinetics of organic anion excretion by the liver in acute intermittent porphyria. Clin. Sci. 38: 677-686, 1970.

Ebert, P. S., Tschudy, D. P., Choudry, J. N., and Chirigos, M. A.: A simple micro method for the direct determination of [^{14}C] δ-aminolevulinic acid production in murine spleen and liver homogenates. Biochim. Biophys. Acta. 208: 236-250, 1970.

Moses, H. L., Stein, J. A., Tschudy, D. P., and Suskind, R. G.: Hepatocellular changes associated with allylisopropylacetamide induced hepatic porphyria in rats. Lab. Invest. 22: 432-442, 1970.

Tschudy, D. P.: Tetrapyrrole tables. In Sober, H. (Ed.): Handbook of Biochemistry. Selected Data for Molecular Biology. Cleveland, Ohio, Chemical Rubber Company, 1971, pp. J249-J260.

Bloomer, J. R., Berk, P. D., Bonkowsky, H. L., Stein, J. A., Berlin, N. I., and Tschudy, D. P.: Blood volume and bilirubin production in acute intermittent porphyria. New Eng. J. Med. 284: 17-20, 1971.

Tschudy, D. P.: The porphyrias. In Conn, H. A. (Ed.): Current Therapy. Philadelphia, W. B. Saunders, Co., 1971, pp. 356-358.

Tschudy, D. P. and Magnus, I. A.: The porphyrias. In Fitzpatrick, T. B. and Scott, E. J. (Eds.): Dermatology in General Medicine. New York, McGraw-Hill Co. In press.

Stein, J. A., Curl, F. D., Valsamus, M. and Tschudy, D. P.: Abnormal iron and water metabolism in acute intermittent porphyria with new morphologic findings. Amer. J. Med. In press.

Serial No. NCI-4004(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Amino Acid Transport and Protein Synthesis

Previous Serial Number: SAME

Principal Investigator: James M. Phang, M.D.

Other Investigator: Ira W. Weiss, M.D.

Cooperating Units: None

Man Years

Total: 5
Professional: 2
Other: 3

Project Description:

Objectives: The aims of this project are to elucidate the membrane phenomena for active transport and to understand regulatory mechanisms for cellular uptake of amino acids and their incorporation into protein.

Methods Employed: Radioisotopically labelled L-amino acids have been used to study in vitro amino acid uptake by fetal rat calvaria and rat kidney cortex slices. To assess protein and collagen synthesis in calvaria, tissues are incubated with L-Proline-^{14}C. Proline is separated from hydroxyproline by thin layer chromatography. These techniques have been used to study the regulatory effects of parathyroid hormone, calcitonin, prostaglandin, cyclic AMP and insulin. Assays of membrane Na^+ - K^+ ATPase and of ATP concentrations are used to assess the mechanisms of these regulatory effects.

Major Findings: We have studied the mechanism of cyclic AMP stimulation of amino acid transport in rat kidney cortex. The synthesis of a mediator appears to be involved: (1) Occurrence of the effect requires at least 2 hours of exposure to dibutyryl cyclic AMP (DBC). After the period of exposure to DBC, tissue transferred to incubation medium without DBC shows undiminished stimulation of amino acid transport. (2) Kinetics studies show an increase in transport V_{max} but no change in K_m. (3) The addition of puromycin and cycloheximide abolishes the DBC stimulation. (4) The stimulation of amino acid transport involves a process dependent on Na-K ATPase. The deletion of sodium or the addition of ouabain blocks the DBC effect.

The above findings suggested the DBC stimulates the synthesis of a transport mediator dependent on Na-K ATPase.

Our studies of the Na-K ATPase isolated from kidney cortex showed: (1) Cyclic AMP has no direct effect on the enzyme. (2) The amount of Na-K ATPase per mg protein was unchanged in DBC-treated tissue. (3) The enzymes from DBC-treated and control tissues showed identical affinities for ATP.

However, the DBC-treated enzyme showed increased affinity for phosphate at Na = 20 mM and K = 5 mM, concentrations of sodium and potassium which approximate the physiologic exposure of the enzyme to these ligands in situ. These studies suggest that cyclic AMP induces the production of a modified Na-K ATPase with a greater affinity for phosphate but unaltered affinity for ATP, thus allowing for accumulation of a high-energy phosphoenzyme complex which participates in active amino acid transport.

We studied the effect of cyclic AMP on amino acid uptake and collagen synthesis in fetal rat calvaria. Using L-proline-U-^{14}C we showed that DBC increases the uptake of proline as well as the incorporation of proline into protein and collagen. The DBC effect is sensitive to medium phosphate concentration. Stimulation of collagen synthesis is maximal at a medium phosphate concentration of 1.2 mM. Higher phosphate concentrations decrease the DBC effect. In fact, at a phosphate concentration of 2.4 mM, DBC produces significant inhibition of collagen synthesis.

The phosphate modification of the DBC effect appears to be mediated through phosphate effects on transport. DBC stimulation of AIB transport was abolished at phosphate concentrations of 2.4 mM. The phosphate effect on transport was not due to phosphate interference with DBC entry. Tissue exposed to DBC in buffer with phosphate concentrations of 2.4 mM and transferred to buffer with phosphate concentrations of 1.2 mM showed undiminished stimulation of AIB transport.

Although phosphate modified the DBC effect on both amino acid transport and collagen synthesis, changes in calcium concentration had no observable effects. Calcium concentrations between 1.2 and 5 mM produced no change in DBC stimulation.

We conclude that cyclic AMP regulation of collagen synthesis is modified by phosphate. The finding suggests that cyclic AMP and phosphate play a physiologic role in collagen metabolism.

Significance to Bio-medical Research: Cell membranes play an important role in the regulation of cellular physiology. Changes in cell membrane function affects cellular metabolism and cell growth. Some of the changes are produced by hormone regulation as a component of normal control mechanisms. Abnormal changes in membrane function may result in uncontrolled neoplastic cell growth. Our studies of the regulation of amino acid transport may elucidate the mechanisms of cell membrane function and derangements of these mechanisms in cancer cells.

Proposed Course: We will continue our studies of the cyclic AMP-mediated changes in Na-K ATPase from kidney cortex. Isolation of the phospho-enzyme complex by millipore filtration will allow us to directly study the characteristics of the Na-K ATPase from cyclic AMP-treated tissues. We also plan to use sucrose density gradients and disc-gel electrophoresis to study the cyclic AMP-modified enzyme.

Since our studies implicate Na-K ATPase as a component in the regulation of amino acid transport, we will study the turnover rates of sodium-dependent and sodium-independent transport mechanisms. We expect that the sodium-dependent mechanism will exhibit rapid turnover.

Using cultured human fibroblasts, we plan to study the regulation of the enzymatic steps in proline biosynthesis. We will emphasize the interaction between cellular mechanisms for transport of exogenous proline and the endogenous biosynthesis of proline. Once this interaction is understood, we hope to identify abnormal interactions in cells from patients with genetic and neoplastic diseases.

Honors and Awards

None

Publications

Phang, J. M., Downing, S. J. and Weiss, I. W.: Cyclic AMP stimulation of amino acid uptake in bone and kidney. Biochim. Biophys. Acta. 211: 605-608, 1970.

Phang, J. M., Finerman, G. A. M., Singh, B., Rosenberg, L. E. and Berman, M.: Compartmental analysis of collagen synthesis in fetal rat calvaria. Biochim. Biophys. Acta. 230: 146-159, 1971.

Hahn, T. J., Downing, S. J. and Phang, J. M.: Insulin effect on amino acid transport in bone: dependence on protein synthesis and Na^+. Am. J. Physiol. In press.

Serial No. NCI-4009(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Study of the Factors Controlling Red Blood Cell Synthesis and Destruction

Previous Serial Number: SAME

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Terrence F. Blaschke, M.D., Joseph R. Bloomer, M.D. and E. Anthony Jones, M.D.

Cooperating Units: None

Man Years:

Total: 4
Professional: 4
Others: 0

Project Description:

Objectives: To study the factors influencing rates of red blood cell production and destruction in normal and pathological states in man, the dog and rat, with particular emphasis on the role of ineffective erythropoiesis in the overall body economy of heme. Studies of hepatic physiology are also conducted as an outgrowth of the use of isotopically labeled bilirubin to quantitate erythropoiesis.

Methods Employed: Erythropoiesis is quantitated by the following techniques: whole blood and red cell volumes are determined with ^{51}Cr-labeled red blood cells; red cell life span is determined with either ^{14}C-glycine incorporation or tritiated diisopropylfluorophosphate labeling; plasma and red blood cell iron turnovers are calculated from the plasma clearance of ^{59}Fe-labeled transferrin, and subsequent incorporation of radio-iron into circulating erythrocytes; ^{14}C-labeled bilirubin and ^{3}H-labeled bilirubin are prepared biosynthetically from delta-aminolevulinic acid; bilirubin production rate, hepatic bilirubin clearance and intercompartmental rate constants in the bilirubin excretory pathway, are determined by analysis of the plasma radio-bilirubin disappearance curves. Ineffective erythropoiesis is estimated from the incorporation of ^{14}C label into fecal urobilinogen during the first ten days after IV administration of ^{14}C-glycine, as well as by computer analysis of the specific activity curve of plasma bilirubin following the administration of labeled heme precursors such as glycine and delta-aminolevulinic acid.

Major Findings: Simultaneous measurements of bilirubin production (BRP) and total red cell volume (TRCV) were performed on 65 occasions in 52 individuals, including normal volunteers and patients with a wide variety of disease states. Since most bilirubin is derived from red blood cell breakdown, a formula was derived for calculating the red cell life span from the data. Red cell survival, calculated from BRP and TRCV correlated well with standard techniques using DFP-^{3}H ($r = 0.98$), ^{51}Cr ($r = 0.86$) and ^{59}Fe ferrokinetics ($r = 0.85$). Since measurement of BRP and TRCV require only 48 hours, this approach may have considerable advantages over red cell labeling techniques requiring 3 weeks to 4½ months.

Simultaneous measurement of BRP, TRCV and mean red cell survival using DFP-^{3}H (LS_{DFP}) has made possible a new approach to the study of ineffective erythropoiesis and other non-erythroid sources of bile pigment. Bilirubin production from red cells was estimated from TRCV and LS_{DFP}, and the result compared with values for total bilirubin production. The non-erythroid fraction of bilirubin production was found to consist of a constant component C of about 0.9 mgm/kg/day and a second component proportional to the erythropoietic rate (proportionality constant $k = .07$). From the values of BRP, C and k, it was possible to estimate the early labeled peak, which averaged 30% of BRP in normal subjects. At accelerated rates of erythropoiesis, the early labeled peak increased in absolute magnitude but decreased when expressed as a fraction of total BRP, averaging 15% when red cell survival was less than 40 days.

Following administration of isotopically labeled δ-aminolevulinic acid, labeled unconjugated bilirubin circulates in the plasma. This labeled bilirubin is formed almost exclusively in the liver. Mathematical techniques have been developed by which the amount of isotopically labeled precursor which has been incorporated into bilirubin can be calculated. Studies have been done in two normal individuals, two patients with red cell aplasia and three patients with acute intermittent porphyria. In the porphyrics the actual amount of bilirubin formed by the liver was calculated by application of the labeled precursor-product relationship to the specific activity of urinary PBG. In porphyrics hepatic bilirubin production ranged from 0.4 - 0.9 mgm/kg/day. Since we have also showed that hepatic bilirubin production in porphyric patients is not increased, similar figures may be applicable to normal subjects. The range derived from this approach is similar to the value for the constant portion of the early labeled peak noted above.

Studies of radiobilirubin kinetics have been performed in additional normal volunteers and patients with a variety of disease states. From these studies, the rates of hepatic bilirubin clearance and bilirubin production were defined for a normal population. Since these are the two principal factors which determine the plasma concentration of unconjugated bilirubin, it was possible to derive, on theoretical grounds, a distribution function for bilirubin concentration in a normal population, and to accurately define an upper limit of normal.

Studies of bilirubin kinetics were performed before and during fasting in humans and rats in order to study the mechanism for the recently described phenomenon of "fasting hyperbilirubinemia." The studies demonstrated that a significant reduction in hepatic bilirubin clearance after 48 or more hours of fasting accounted for this phenomenon.

The effect of glutethimide, a hypnotic structurally related to phenobarbital, has been examined in a group of patients with Gilbert's Syndrome and normal volunteers. Glutethimide, given as 500 mgm p.o. q.h.s., produced a significant reduction in the plasma concentration of unconjugated bilirubin in all subjects. In Gilbert's Syndrome, initial values of 1.7 to 0.5 mgm% fell to normal within one week, and averaged 30% of baseline after 2 weeks. Similar reductions occurred in the normal volunteers. Studies with labeled bilirubin, both before and during therapy, indicated that the fall in plasma bilirubin concentration resulted from both an increase in hepatic bilirubin clearance and a decrease in bilirubin production. An increase in bilirubin clearance and a reduced plasma bilirubin concentration, but no fall in bilirubin production, was observed in phenobarbital treated patients. In contrast to its effects on bilirubin, glutethimide was found to produce no change in the initial disappearance rates of either bromsulfalein (BSP) or indocyanine green.

As part of a broad study of various aspects of hepatic function in Gilbert's Syndrome, detailed studies of BSP kinetics were performed in 8 patients. These studies revealed a previously unreported abnormality in the pattern of BSP clearance in this condition. Since BSP is excreted into the bile conjugated with glutathione, rather than glucuronic acid, theories attributing jaundice in Gilbert's Syndrome to a defect in glucuronide conjugation may need re-examination.

Significance to Bio-medical Research: Studies with isotopically labeled bilirubin have produced the following useful results: (1) determination of the bilirubin production rate for a wide variety of disorders, (2) an estimation of the mean red cell life span, both in normal individuals and individuals with hemolysis, (3) delineation of the defect in bilirubin metabolism in Gilbert's Syndrome, (4) establishing of the normal range for the plasma unconjugated bilirubin concentration, (5) a method by which the hepatic clearance of bilirubin can be determined at all plasma unconjugated bilirubin concentrations. Studies of hepatic bilirubin production in patients with porphyria have provided the first measurement of this parameter in man, and have led to further insights into the biochemical defect in this disease. Studies with Doriden® and phenobarbital are leading to a better understanding of the mechanisms by which the liver metabolizes drugs and responds to exogenous materials.

Proposed Course: Measurements of hepatic bilirubin production will be extended to include normals and patients with various liver diseases. The basic mechanisms underlying hepatic enzyme induction by drugs will be investigated. Further studies of organic anion excretion in Gilbert's Syndrome will be pursued.

Honors and Awards

None

Publications

Berk, P. D., Bloomer, J. R., Howe, R. B., and Berlin, N. I.: Constitutional hepatic dysfunction (Gilbert's Syndrome): A new definition based on kinetic studies with unconjugated radio-bilirubin. Amer. J. Med. 49: 296-305, 1970.

Bloomer, J. R., Berk, P. D., Howe, R. B., Waggoner, J. G., and Berlin, N. I.: Comparison of fecal urobilinogen excretion with bilirubin production in normal volunteers and patients with increased bilirubin production. Clin. Chim. Acta. 29: 463-471, 1970.

Berk, P. D., Bloomer, J. R., Howe, R. B., and Berlin, N. I.: The life span of the red cell as determined with labeled bilirubin. In Jamieson, G. A. and Greenwalt, T. J. (Eds.): The Formation and Destruction of Blood Cells (American National Red Cross Third Annual Scientific Symposium). Philadelphia, J. B. Lippincott, 1970, Chapter IV.

Berk, P. D., Bloomer, J. R., and Berlin, N. I.: Y and Z: New terms in the bilirubin equation. (Editorial). New Eng. J. Med. 283: 1166, 1970.

Barth, R. F., Grimley, P. M., Berk, P. D., Bloomer, J. R., and Howe, R. B.: Excess lipofuscin accumulation in constitutional hepati dysfunction. A. M. A. Arch. Path. 91: 41-47, 1971.

Bloomer, J. R., Berk, P. D., Bonkowsky, H. L., Stein, J. A., Berlin, N. I., and Tschudy, D. P.: Blood volume and bilirubin production in acute intermittent porphyria. New Eng. J. Med. 284: 17-20, 1971.

Bloomer, J. R., Berk, P. D., Howe, R. B., and Berlin, N. I.: Bilirubin metabolism in congenital nonhemolytic jaundice. Ped. Res. In press.

Bloomer, J. R., Berk, P. D., Howe, R. B., and Berlin, N. I.: Interpretation of plasma bilirubin levels based on studies with radiobilirubin. J. Amer. Med. Assoc. In press.

Serial No. NCI-4013(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Calcium Metabolism in Malignant Disease and Metabolic Disease of Bone

Previous Seiral Number: SAME

Principal Investigator: James M. Phang, M.D.

Other Investigators: Ira W. Weiss, M.D. and Mones Berman, Ph.D.

Cooperating Units: Office of Mathematical Research, NIAMD

Man Years

Total: 6
Professional: 3
Others: 3

Project Description:

Objectives: This project is designed to investigate calcium metabolism in normal man as well as in patients with neoplastic and metabolic bone disease. Physiologic and pathophysiologic regulatory mechanisms have been emphasized.

Methods Employed: Serum ^{47}Ca disappearance and excretion of ^{47}Ca into urine and feces are combined with metabolic balance data in a computer multicompartmental analysis to give steady state rate constants and numerical values for calcium metabolic parameters. The physiologic hypotheses suggested by this analysis are tested in animal and in vitro experiments and in additional studies of calcium metabolism in man. Where applicable, parathyroid hormone assays, bone histology, urinary hydroxyproline determinations and bone X-ray crystallography are obtained.

Major Findings: We have investigated the effects of diphosphonates on various diseases in man. Diphosphonates, a family of compounds with structural similarities to pyrophosphate, inhibit both calcification and bone resorption. Unlike pyrophosphates, diphosphonates are resistant to enzymatic hydrolysis by pyrophosphatase. In patients with myositis ossificans progressiva and calcinosis universalis, diphosphonates produce marked clinical improvement. ^{47}Ca kinetics and balance studies show decreased bone calcium turnovers and decreased hydroxyproline excretion in patients on the drug. Calcium balance, however, is unaffected. Thus, the pharmacologic effect occurs without demineralization of normal

skeletal bone. Measurements of bone density using a Nordlund-Cameron densitometer corroborates the findings from balance studies. Patients on diphosphonates show increased rather than decreased skeletal bone mass.

The above findings suggest that diphosphonates are useful in the treatment of patients with pathologic calcifications.

Significance to Bio-medical Research: The maintenance of calcium homeostasis is vital to man. Small changes in plasma calcium ion activity profoundly disturbs many physiologic processes. In cancer patients with bony metastases, hypercalcemia may occur because bone is a major tissue component in the calcium homeostatic system. Our studies using pharmacologic agents which affect the calcification process will elucidate the mechanisms of calcification and provide a therapeutic approach in treating bony metastases, metabolic bone diseases and disorders of calcium homeostasis.

Proposed Course: We will continue the studies of diphosphonate effects in man. We will emphasize the effects of the drug in two groups of patients: (1) Patients with pathologic calcification such as myositis ossificans progressiva and calcinosis universalis. (2) Patients with idiopathic osteoporosis.

Honors and Awards

None

Publications

Kales, A. N. and Phang, J. M.: Dietary calcium perturbations in patients with abnormal calcium deposition. J. Clin. Endo. Metab. 31: 204-212, 1970.

Ramberg, C. F., Jr., Phang, J. M., Mayer, G. P., Norberg, A. I. and Kronfeld, D. S.: Inhibition of calcium absorption and elevation of calcium removal rate from bone in fluoride-treated calves. J. Nutr. 100: 981-990, 1970.

Ramberg, C. F., Jr., Mayer, G. P., Kronfeld, D. S., Phang, J. M., and Berman, M.: Calcium kinetics in cows during late pregnancy, parturition, and early lactation. Am. J. Physiol. 219: 1166-1177, 1970.

Kales, A. N. and Phang, J. M.: The effect of divided calcium intake on calcium metabolism. J. Clin. Endo. Metab. 32: 83-87, 1971.

Weiss, I. W., Fisher, L., and Phang, J. M.: Diphosphonate therapy in myositis ossificans progressiva. Ann. Int. Med. In press.

Phang, J. M. and Weiss, I. W.: The maintenance of calcium homeostasis in man. Invited chapter in Handbook of Physiology. G. D. Aurbach (Ed.) In press.

Serial No. NCI-4014(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Immune Response in Normal and Pathological States

Previous Serial Number: SAME

Principal Investigator: Warren Strober, M.D.

Other Investigators: Thomas A. Waldmann, M.D., Z. M. Falchuk, M.D., and Charles I. Heller, M.D.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMD

Man Years

Total: 5
Professional: 4
Other: 0

Project Description:

Objectives: Studies were directed toward determining the nature and mechanism of circulating and cellular immune responses to antigenic challenge in animals and humans, including patients with immune deficiency states, such as ataxia telangiectasia, agammaglobulinemia, intestinal lymphangiectasia and gluten-induced enteropathy. Insight into the cause of the high incidence of reticuloendothelial malignancies associated with these disorders was sought.

Methods Employed: Antibody responses in the individual immunoglobulin classes was measured using a modification of the C'1a fixation and transfer test of Borsos and Rapp. This test allows simultaneous quantitation of antibody in each of the immunoglobulin classes through the use of a quantitative C'1a fixation reaction. IgM antibodies are measured in a direct fixation reaction while IgG and IgA antibodies are measured in an indirect reaction using enhancing anti-antibodies specific for IgA and IgG.

The C'1a fixation and transfer test was used to measure antibody responses to arsanilic acid coupled with bovine serum albumin, fluid BSA and keyhole limpet hemocyanin. Local gastrointestinal immune responses were assessed by measuring mucosal immunoglobulin synthesis and mucosal antibody synthesis using a solid phase immunoabsorbant technique.

In vivo antibody responses in patients with ataxia telangiectasia, Wiskott-Aldrich syndrome and other immune deficiency states were assessed by measuring antibody responses to Blood Group Substance, Vi antigen, Tularemia antigen and Pneumococcal Polysaccharide. In vivo delayed hypersensitivity was assessed with DNCB skin tests and homologous skin grafts.

Major Findings: 1. Studies of the nature of immune response in experimental animals. In previous studies it was shown that IgA antibody responses to the antigen arsanilic acid (AA) coupled to carrier bovine serum albumin (BSA) was greatly diminished in neonatally thymectomized animals at 8 weeks of age. In recent studies this finding was extended to show that the diminution in responses was related to the nature of the carrier protein. Thus, responses to AA on keyhole limpet hemocyanin (KLH) carrier were normal insofar as IgA antibody response was concerned. Since KLH is a less thymic dependent antigen than BSA we have interpreted this finding to mean that IgA antibody responses are uniquely dependent on thymic dependent helper cells. Further studies were done on the factors controlling IgA antibody responses. It was found that fluid BSA evoked no IgA when given by the intravenous route. However, when given via the footpad in complete Freund's adjuvant very large IgA responses were obtained. Similarly, responses to intravenous arsanilic acid on BSA evoked a relatively small IgA antibody response whereas footpad injection of the same antigen together with complete Freund's adjuvant led to a large anti-arsanilic acid IgA antibody response. Finally, chemical denaturation of BSA (by diazotization reaction with arsanilic acid) led to IgA responses even when BSA is given intravenously. These studies, taken with the studies of the pattern of IgA responses in thymectomized animals, support the concept that an auxillary cellular system is necessary for the production of IgA responses. This cellular system involves macrophages or thymus-dependent lymphocytes or both.

2. Studies of immune deficiency states: a. Ataxia telangiectasia. The C'1a fixation and transfer test was applied to the studies of antibody responses to group substance in patients with ataxia telangiectasia. When antibody responses to group substance was studied it found that those patients lacking IgA, as expected, also lacked IgA antibody responses. Of greater interest was the fact that even those few patients having IgA immunoglobulin in the serum still failed to produce IgA antibody with group substance specificity. Since all patients with ataxia telangiectasia and immunologic deficiency also have thymus gland abnormalities, a poor response in IgA immunoglobulin class may indicate that IgA responses and thymus abnormalities are closely linked in these patients. Indeed, this is suggested by studies in rabbits mentioned above.

b. Gluten-sensitive enteropathy. In association with Dr. Z. M. Falchuk studies of the local immune responses in gastrointestinal diseases were continued. A method was developed for the measurement of immunoglobulin production by specimens of intestinal mucosa obtained by biopsy and cultured in vitro. This method depends on the use of the solid phase

immunoadsorbant bromacetyl cellulose coupled with anti-immunoglobulin (BAC-anti-Ig). The biopsy specimen is cultured in the presence of ^{14}C-leucine; the amount of incorporation into immunoglobulin is measured after several hours by specific binding of labeled immunoglobulin present in homogenates of the cultured biopsy to the solid phase immunoadsorbant.

Using this method patients with hypogammaglobulinemia and agammaglobulinemia have low or absent mucosal immunoglobulin production. On the other hand patients with active gluten-induced enteropathy have greatly elevated levels of gut immunoglobulin production. This is a direct demonstration of increased local immunoglobulin synthesis in gluten sensitive enteropathy patients previously suggested by immunogluorescent studies.

A method for the measurement of local mucosal antibody production was also devised. This makes use of another solid phase immunoadsorbant, sepharose coupled with antigen. Again biopsies of intestinal mucosa are cultured in the presence of ^{14}C leucine and the homogenates of these biopsies are exposed to the solid phase immunoadsorbant. Newly synthesized antibody binds specifically to antigen on the adsorbant and can be subsequently eluted and quantified. Using this method it was determined that patients with gluten-induced enteropathy in remission produce anti-gliadin antibodies following reintroduction of gluten into the diet. The amount of anti-gliadin produced ranges from 33-70% of the total increase in IgA and IgM immunoglobulin synthesis; hence anti-gliadin production is a quantitatively important process. The demonstration that anti-gliaden antibodies are produced at mucosal sites soon after reintroduction of gluten into the diet of patients with gluten-induced enteropathy greatly strengthened the hypothesis that immunologic factors play an important part in the pathogenesis of this disease.

Significance to Bio-medical Research: The IgA immunoglobulin system is quantitatively the most important immunoglobulin present at mucosal surfaces. As such, IgA responses are important in the protection against a variety of infectious diseases, and may also function in preventing the development of autoimmune disease. The studies with the C'1a fixation and transfer test have so far shown that the IgA immunoglobulin system may be more dependent on an auxillary cellular system than other immunoglobulin systems. This may explain the fact that here is an association between IgA deficiency and thymic abnormalities in patients with ataxia telangiectasia. Of more general interest, the relationship of IgA responses to a thymic-dependent cellular system may also explain the fact that autoimmune disease occurs with a greater frequency in individuals who lack the IgA immunoglobulin system.

Studies of the responses of a local gastrointestinal immune system have shown that local immunoglobulin responses may mediate the development of gastrointestinal disease. These studies provide important techniques for the studies of a large variety of gastrointestinal diseases in where an immunological basis is suspected. More particularly, the studies have led to a greater understanding of the local immune response occurring

gluten-induced enteropathy after gluten challenge and strongly suggest that immunological factors play a role in the pathogenesis of this disease.

Studies of the immune responses in patients with ataxia telangiectasia, and other immune deficiency states are leading to a greater understanding of the site and molecular nature of the defect in these various diseases. Patients with immune deficiency are more prone to develop neoplasms and it is therefore probable that immune processes constitute an important defense against the development of such neoplasms. Studies of the immune defects encounted in immune deficiency diseases will provide a greater understanding of how immune processes prevent the development of neoplasms.

Proposed Course: Studies of the factors which control the IgA response will be continued. An attempt will be made to delineate the role of specific cellular systems in the IgA response. In this regard antigen associated with macrophages will be used to stimulate the antibody response in attempt to see whether macrophages play a necessary role in the development of IgA responses. In addition IgA responses in relation to carrier-dependent immune reactions will be studied in an attempt to show that IgA responses are dependent upon the normal functioning of a thymus-dependent lymphocyte population.

In humans, the C'1a fixation and transfer test will be used to study patterns of response in ataxia telangiectasia, and other immune deficiency states to a variety of antigens in order to relate these disorders to animal models of immunologic deficiency.

Patients with Wiskott-Aldrich syndrome, ataxia telangiectasia and other immune deficiency states will continue to be studied from a broad aspect. Attempts will be made to relate these immune deficiency states to animal models of immune deficiency such as thymectomized rodents, bursectomized birds and animals treated with various drugs or antiserums.

Continued studies of the role of immunologic factors in gluten-induced enteropathy and other gastrointestinal diseases will be carried out. In particular, an attempt will be made to show that locally produced antigluten antibodies are directly toxic to mucosal epithelial cells in long term tissue culture. Patients with various forms of immune deficiency will be studied with their capacity to produce a local immunoglobulins. In this regard, an attempt will be made to study the pattern of intestinal immunoglobulin synthesis in a variety of dysgammalgobulinemia.

Honors and Awards

None

Publications

Loeb, P. M., Strober, W., Falchuk, A. M., and Laster, Leonard:

Incorporation of L-Leucine-^{14}C into immunoglobulins by jejunal biopsies of patients with celiac sprue and other gastrointestinal diseases. J. Clin. Invest. 50: 559-569, 1971.

McFarlin, D. E., Strober, W., Barlow, M., and Waldmann, T. A.: The immunological deficiency state in ataxia telangiectasia. Research Publication of the Association for Research in Nervous and Mental Disease. In press.

Clough, J. D., Mims, L. H. and Strober, W.: Deficient IgA antibody response to arsanilic acid-BSA in neonatally thymectomized rabbits. J. Immunol. In press.

Serial No. NCI-4015(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Development and Function of Humoral and Cellular Mechanisms of Host Defense

Previous Serial Number: NONE

Principal Investigator: R. Michael Blaese, M.D.

Other Investigators: Thomas Waldmann, M.D., Warren Strober, M.D., Paul Weiden, M.D., J. J. Oppenheim, M.D., J. Wunderlich, M.D., E. Rosenberg, M.D., and R. Jacobs, M.D.

Cooperating Units: Immunology Branch, NCI, and Laboratory of Microbiology, Immunology Section, NIDR

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: The objectives of this study were to determine mechanisms important to the development and function of both the humoral and cellular immune response in animals and man; to determine the nature of the deficiency in immune function characterizing such disease states as the Wiskott-Aldrich syndrome, intestinal lymphangiectasia and advanced malignancy; and, to develop approaches to prophylaxis and/or therapy of the infectious and neoplastic disease associated with defective immune processes.

Methods Employed: Antibody responses of experimental animals and man were determined after immunization with bacterial vaccines such as brucella abortus, S. typhosa, and pertussis; with particulate cellular antigens such as sheep, burro, and chicken RBC's; with purified protein antigens such as KLH, diphtheria and tetanus toxoids, guinea pig γ globulin and bovine γ globulin; and with purified polysaccharide antigens such as pneumococcal polysaccharides types I, II, III, and the V_i antigen of E. Coli. The antibodies were detected by standard techniques or by newly developed micro methods of bacterial agglutination, red cell hemolysis, and passive hemagglutination using $CrCl_3$ as an agent to couple the antigens to a carrier RBC surface. In addition, modifications

of the Farr antigen binding assay were used.

Cellular immune responses were measured by delayed hypersensitivity skin tests, contact sensitivity to DNCB, and skin allografts. Lymphocyte transformation in vitro was carried out utilizing non specific mitogens such as PHA, PWM and staph filtrate; specific antigens such as SLO, SKSD, diphtheria and tetanus toxoids, KLH and candida albicans; and allogeneic cells in mixed leukocyte culture. Lymphocyte function in vitro was also measured by production of mediators of cellular immunity such as lymphotoxin and MIF, and by cytotoxicity reactions with antibody sensitized target cells.

Major Findings: (1) Studies of the development of immune compentence were carried out in neonatal rats. We have previously demonstrated that the ability of neonates to produce specific antibody following antigenic challenge develops in a distinct stepwise fashion and over a period of the first 4 weeks of life. It was shown that this apparent development of the antibody forming system of cells was actually a function of the maturation of the antigen processing system of macrophages. The apparent immune deficiency in the neonate in response to many antigens could be corrected by treating the neonates with mature adult macrophages, thus allowing a specific immune response to occur earlier in life. The immmaturity of the antigen processing macrophage system of cells also favors the more ready development of immunologic tolerance in the neonate, which can also be prevented by mature macrophage treatment.

It was found that the state of maturation or activation of the neonates' macrophages could be enhanced by a variety of materials. BCG, brucella, typhoid and pertussis vaccines; endotoxins from E. coli, Serratia marcescens or Salmonella typhosa; alum, silica and poly IC; all known to function as immunologic adjuvants, were capable of macrophage activation and thus allowed the neonate to become more immunologically responsive. Similarly macrophage activation in the neonate by these substances will make these animals resistant to the induction of immunologic tolerance.

Cellular immune responses in the neonate are also dependent upon macrophage maturation and the development of this form of immunity was studied utilizing Listeria monocytogenes infections. Listeria are killed by mature or activated macrophages, and therefore this infection provides a sensitive assay for the activation status of macrophages. It was shown that the mature macrophage will protect the neonate from infection with this organism and that agents which are capable of activating the afferent limb, antigen processing, functions of the macrophage will also activate the neonate's macrophages for this efferent limb bactercidal function. Thus, it was demonstrated that the macrophage plays an important dual role in immune responses, both as the antigen processing cell essential for the initiation of immune responses to many antigen and also as the ultimate effector cell in immune reactions responsible for killing

invading bacterial organisms and probably also essential for the elimination of malignant cells.

(2) Studies of the role of the central lymphoid organs in the development of immune responsiveness were continued. Agammaglobulinemic chickens produced by surgical removal of the Bursa of Fabricius, combined with sublethal radiation in the immediate post hatching period, were studied for the ability for various cell populations to reconstitute normal humoral immune responsiveness. It was found that normal spleen, but not normal bone marrow or normal bursal lymphocytes, could reconstitute immunoglobulin production in these agammaglobulinemic birds. In addition lethally irradiated chickens protected by bone marrow infusions became agammaglobulinemic if the bone marrow donors were also agammaglobulinemic. This indicates that the central lymphoid organ responsible for the development of the humoral immune system, the bursa of Fabricius has only a limited period of time during which it is capable of inducing stem cells to differentiate into a system of cells involved in humoral immunity. A situation which is embryologically reminiscent of the requirement of an optic vessicle for the induction or differentiation of lens tissue in the eye. When the inducing substance is placed in a developing eye at a time later than that at which the lens would ordinarily develop, it is no longer capable of inducing differentiation of the lens. These observations may have important implications for the potential reconstitution of patients with immune deficiency characterized by hypogammaglobulinemia by simple bone marrow transplantation.

Characterization of a sporadic anemia occurring in these agammaglobulinemic chickens has demonstrated marked hemolysis with shortened survival of agammaglobulinemic anemic red cells tagged with H^3-DFP in both agamma and normal chickens. However, normal red cells had a normal survival in agammaglobulinemic chickens. Our data indicate an acquired defect of the agammaglobulinemic red cell itself, which probably occurs prior to the release of these cells into the circulation. False positive Coomb's tests were demonstrated, which showed agglutination of the agammaglobulinemic anemic red cells by a natural antibody present in normal rabbit and sheep serum. This antibody reacted with some antigenic determinant specific to the agammaglobulinemic anemic red cell surface, but this was not an immunoglobulin or other serum protein. These birds provide an unusual model for an "autoimmune like" disease without demonstrable autoantibodies, and thus they provide insight into the basic mechanism of such disorders.

Techniques for the in vitro study of lymphocyte transformation in chickens were developed for additional studies of cell mediated immunity. It was demonstrated that lymphocyte blastogenic responses to specific antigens and non-specific mitogens such as phytohemagglutinin were dependent upon an intact thymus system but were normal in the absense of a bursa of Fabricius. In addition specific antibody was effective in inhibiting the blastogenic response of sensitized lymphocytes from both normal chickens and agammaglobulinemic chickens. This data reinforces our

concept of the functional separation of the thymus dependent cellular immune system and the thymus independent humoral immune system, and demonstrates one mechanism of feedback control by the humoral immune system on cellular immune reactions.

(4) Studies of immune deficiency disease in man were continued. Part of the clinical evaluation of patients for immune competence includes determination of cellular immune reactivity. This includes skin tests to determine the status of delayed hypersensitivity possessed by an individual, and studies of the responses of lymphocytes in vitro to a variety of mitogenic agents. One of these mitogenic agents, phytohemagglutinin, has become a standard for the comparison of responsiveness of individual patients. We initiated a study to evaluate the usefulness of phytohemagglutinin as a skin test in evaluating patients with immune deficiency. The advantages using phytohemagglutinin in this manner would be the simplicity and speed (would not require tissue culture techniques) and its more ready application to small children in which suitable quantities of blood for tissue culture may be quite difficult to obtain. It also has the distinct advantage of being able to measure delayed hypersensitivity cell mediated responsiveness in vivo which would be independent of the previous antigenic exposure of the individual being tested. About 85 normal individuals and patients with a variety of immune deficiency diseases have now been studied. Normal people develop an area of erythemia and induration of approximately 10-15 mm in diameter within 24 hours after intradermal injection of 2 μg of phytohemagglutinin. This response is quite similar in its characteristic to a classical delayed type hypersensitivity reaction and histologically is characterized by round cell infiltration and perivascular lymphocyte cuffing. There is also some invasion by polymorphonuclear leukocytes and a few eosinophils. We have found that a majority of patients with clinical immune deficiency and anergy also respond to a similar dose of phytohemagglutinin. That is patients with Hodgkin's disease, advanced metastic malignancy, intestinal lymphangiectasia, and lymphocarcoma respond in a normal fashion. However, some patients with the Wiskott-Aldrich syndrome appear to be hyporesponsive to intradermal phytohemagglutinin and two patients that we have had the opportunity to study with severe combined immune deficiency or the Swiss type agammaglobulinemia have shown no response to as much as 10 μg of phytohemagglutinin administered intradermally. Thus, the PHA skin test is probably too sensitive for routine evaluation of cell mediated immunity in patients, but may be of some value in determining the absolute integrity of the thymic dependent immune system in severe immune deficiency.

Studies of cell mediated immunity in patients with intestinal lymphangiectasia were also continued. This disease is characterized by hypoproteinemia and edema secondary to massive gastrointestinal protein loss. Pathologically, these patients have markedly dilated lymphatics in the intestinal wall which can actually be demonstrated to leak lymph

directly into the bowel lumen. The patients also have lymphocytopenia as a result of this loss of lymph with its cohort of lymphocytes. Previous studies have shown these patients to have relatively marked anergy with inability to manifest delayed type skin tests and inability to develop contact sensitivity to dinitrochlorobenzene (DNCB) and these patients may accept skin allografts for a period of several years. We studied the ability of peripheral blood lymphocytes in these patients to respond in tissue culture to a variety of mitogenic agents. It was found that the patients have markedly depressed responses to non-specific mitogens such as phytohemagglutinin, pokeweed mitogen and staph filtrate. They also have depressed responses to specific antigens and allogeneic cells in mixed leukocyte culture. The degree of depression of in vitro lymphocyte reactivity seems to correlate generally with magnitude of gastrointestinal protein loss as revealed in the serum albumin concentration, and it was postulated that the loss of the recirculating pool of lymphocytes into the bowel was responsible for the anergy observed. Because of the generalized lymphatic abnormalities in these patients they frequently develop leaking lymphatics along the serosal surfaces of their intestinal tract as well as into the bowel lumen. These leaking lymphatics result in chylous effusions. In studying the lymphocyte responsiveness in vitro of cells obtained from these chylous effusions, we observed that these cells are normally responsive in vitro to the various mitogenic agents. Thus, the recirculating population of small lymphocytes which ordinarily traverse the efferent lymphatics and the thoracic duct to return to the circulation, do contain those cells which are capable of blastogenic responses to the various mitogens. Our patients have deficient lymphocyte responses in their peripheral blood because the blood has been depleted of this recirculating population of cells which are lost into bowel.

In association with Drs. John Wunderlich and Eugene Rosenberg of the Immunology Branch of the NCI, additional studies of cellular immune responses in immune deficiency patients were carried out. In these studies a serum antibody termed LDA or lymphocyte dependent antibody was found which would recruit non immune lymphocytes for specific cellular cytotoxic reactions. Drs. Wunderlich and Rosenberg have characterized this antibody as 7S IgG antibody which is specific for the HL-A histo compatibility antigens found on normal human lymphocytes. The antibody is specific for the target cells which it coats, but the lymphocytes which it recruits for the cytotoxic reactions are not immune to the target cell. Therefore, this antibody could be used as another means of testing the lymphocyte function in man.

Clinical evaluation of cellular immune competence was compared with in vitro lymphocyte transformation responses to non-specific mitogens, specific antigens, and allogeneic cells in mixed leukocyte culture, and these were compared with the cytotoxic responses of these patients' lymphocytes to lymphocyte dependent antibody coated target cells. Patients with the Wiskott-Aldrich syndrome, a disease characterized by thrombocytopenia, eczema and multiple infections, have marked clinical anergy

with inability to manifest delayed hypersensitivity skin tests, they cannot develop contact sensitivity to DNCB and they reject skin homografts very poorly. The in vitro transformation of lymphocytes from these patients show an unusual pattern of responsiveness with normal responses to non-specific mitogens but extremely defective responses to specific antigens and allogeneic cells. However, the cytotoxicity reactions in these patients recruited by lymphocyte dependent antibody were completely within the normal range. Thus, in these patients there is a marked discordance between clinical evaluation of cellular immune responsiveness by skin tests and skin homografts with the in vitro responses of their lymphocytes to non-specific mitogens and to LDA recruited cytotoxicity reactions.

Another group of patients studied were those with anergy associated with advanced malignant disease. These patients had completely defective cellular immune responses by clinical evaluation also had depressed in vitro lymphocyte responses to all classes of mitogens and yet they also had lymphocytes with normal cytotoxic potency when tested by the lymphocyte dependent antibody technique. An additional group of patients studied were those with intestinal lymphangiectasia. As mentioned previously, these patients are characterized by hypoproteinemia secondary to profound gastrointestinal protein loss which is also associated with the loss of lymphocytes into the bowel. Lymphocyte transformation to all classes of mitogens in these patients are significantly depressed, but lymphocyte dependent antibody mediated cytotoxic reactions were greater than normal in every case, suggesting that separate populations of lymphocytes are responding to the mitogenic stimuli from those responding to the LDA by cytotoxicity reactions. In confirmation of this suggestion, it was shown that although peripheral blood responses to mitogens were impaired while chylous effusion blastogenic responses were normal, the converse was true for LDA cytotoxicity reactions. Thus, while cytotoxic reactions recruited by lymphocyte dependent antibody were normal in peripheral blood, they were significantly depressed chylous effusion lymphocytes. Therefore, it appears reasonable that the long-lived recirculating pool contains those lymphocytes responsible for blastogenic responses to non specific mitogens, specific antigens and allogeneic cells and the non-recirculating, short-lived pool of lymphocytes contains those cells which are responding by cytotoxic reactions to LDA coated target cells. This clear separation of lymphocyte function by characteristics of life span and migration pattern within the body may provide us with new mechanisms by which we may manipulate the immune system to the benefit of patients with immune deficiency and malignant disease.

Significance to Bio-medical Research: The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune system in animals and man. They indicate that disorders in immune function may be related to the absence or malfunction of such central lymphois organs as the thymus or bursa of Fabricius or to immaturity of such auxillary

systems as the macrophage required for the processing of antigens in the initiation of an immune response. They also indicate that the macrophage plays multiple roles in a normal immune response, being both important in the initiation or afferent limb of such responses and also as the ultimate effector cell in many immune reactions. Our studies on macrophage activation provide, we think, an explanation for the mechanism by which such agents as BCG have shown some therapeutic effectiveness in the treatment of cancer. That is, BCG can activate macrophages for more effective recognition of antigens and thereby promote a more efficient immune response, BCG will also activate the efferent limb killing function of the macrophage to allow a more efficient destruction of the malignant cell. In addition, the ability to manipulate the degree of activation of macrophages in the neonate may provide us with the mechanism of non specific prophylaxis of malignant disease caused by vertically transmitted tumor viruses which ordinarily induce tolerance when exposed to the neonatal animal. Our studies of lymphocyte function in patients with immune deficiency and advanced malignancy have extended our understanding of the nature of the reactions produced by different classes of lymphocytes and the observation that some of these functions may remain intact in severely anergic individuals may provide us with a mechanism of approaching effective immunotherapy of malignancy occurring even in severely immune deficient patients.

Proposed Course: Continuing studies directed toward understanding the normal pathways involved in the development and function of the immune system and application of this new knowledge in approaches to the prevention or therapy of malignant disease.

Honors and Awards

Election to the American Association of Immunologists

Publications:

None

SUMMARY REPORT

RADIATION BRANCH

NATIONAL CANCER INSTITUTE

July 1, 1970, through June 30, 1971

The past year has been the most important period in the history of the Radiation Branch from the standpoint of clinical investigation. As briefly summarized in the following paragraphs, significant contributions have been made to the understanding of and ability to effectively treat several types of human cancer and these results have gained widespread recognition throughout the world as witnessed by an ever increasing flow of visitors, both national and foreign, to our department.

A prominent highlight has been the continued maturing of our long term study on Hodgkin's disease, to which acquisition of patients was completed in 1969. This research investigation has provided new insights into the biologic behavior of the disease, the methods for proper evaluation of individual clinical presentations, the most suitable therapeutic approaches as defined by prospective comparison of treatment regimes, and lastly but not least into techniques for therapy which have markedly attenuated the normal tissue complications of irradiation. The five-year mortality rate from combined disease and treatment complications has been less than 10% for patients with localized or regionally advanced Hodgkin's disease treated with extensive prophylactic irradiation. This represents a significant improvement over both concurrent controls and results reported from other medical centers. With the establishment of early to regionally advanced Hodgkin's disease as one of the amenable forms of cancer to curative treatment, an educational program has been sponsored by the Radiation Branch for practicing physicians. During the past year, eighty radiologists and their colleagues in allied disciplines such as physics have received formal "in-house" training in the radiotherapeutic management of Hodgkin's disease.

The concept of early administration of systemic chemotherapy for cancers which have a propensity for early hematogenous dissemination has received considerable impetus from a research program involving Ewing's sarcoma. This highly malignant bone cancer typically manifests the rapid development of metastatic disease in 80-90% of patients within two years of diagnosis. For the 26 patients receiving prophylactic chemotherapy in our study during the past seven years, the two-year disease free rate has been increased to 50% with a corresponding improvement in survival. These observations form the basis for a general principle applicable to many forms of human cancer, namely the use of empiric chemotherapy as an adjuvant to the radiologic or surgical treatment of the primary tumor site.

Investigations on the lymphoproliferative disorders of lymphocytic lymphoma and chronic lymphocytic leukemia have been greatly accelerated and now

constitute a major research effort in the Radiation Branch. The promising pilot studies described in previous annual reports have been expanded into more intensive evaluation and the use of new radiotherapy techniques including total body irradiation and total nodal irradiation offer the prospect of developing into an important "breakthrough" within the next several years.

Several new areas of clinical research have been instituted, including the use of radiation therapy to stabilize or reverse the growth hormone deficiency and diabetes insipidus in patients with Hand-Schuller-Christian disease by irradiation of the hypothalamic-pituitary region. Several studies aimed at chemical radiopotentiation have commenced which are concerned with the treatment of high-grade gliomas of the central nervous system and carcinomas of the upper respiratory tract. In addition, the application of computer technology to radiation therapy has continued and plans are being evolved to automate the technical aspects of treatment administration within the near future.

The service responsibilities, both with respect to clinical consultations and non-clinical irradiation of biologic materials and animals, has continued to increase. A total of 228 patients were formally seen in consultation and advice regarding management was provided on numerous other patients on an "ad hoc" basis. Overall, 4240 individual treatments and 3368 non-clinical radiation exposures were performed during the past year, both these figures representing significant increases compared to the previous year. This work load, combined with an extremely active clinical follow-up and multiple technical difficulties associated with the new high energy linear accelerator, has resulted in a demanding year for the relatively small senior staff, consisting of two radiation therapists and three radiation physicists.

Serial No. NCI-958(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Radiotherapeutic Management of Hodgkin's Disease

Previous Serial Number: Same

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: Frederick L. Faw, M.S., Dwight W. Glenn, M.S., and Thomas C. Pomeroy, M.D.

Cooperating Units: None

Man Years:

Total: 4/2
Professional: 4/2

Project Description:

Objectives: The study has included the investigation of (a) established and new diagnostic techniques for their value in determining the anatomic extent of involvement with disease and usefulness in guiding treatment, (b) various treatment approaches by prospective comparison, and (c) new technical methods for administration of wide-field irradiation with improved homogeneity of absorbed dose.

Patient Material: A total of 164 patients with previously untreated Hodgkin's disease were entered into this study between January, 1965, and December, 1969.

Methods Employed: Prior to treatment, extensive diagnostic evaluation was performed in an attempt to (a) evaluate the anatomic extent of disease using the most sophisticated available methods, and to (b) determine the evaluation of the appropriateness of these diagnostic studies in guiding decision-making. Patients were randomly assigned to various treatment options and carefully followed after completion of treatment to establish the relative merit of different treatment approaches, to gain information on the patterns and sites of recurrence as correlated with several base-line characteristics of the disease, and to document the complications resulting from different techniques used in irradiating these patients.

Major Findings: As described in detail in the publications listed, this study has scientifically demonstrated for the first time the requirement for extensive prophylactic irradiation of clinically uninvolved lymph node areas in the management of Hodgkin's disease. Patients treated with standard techniques have had survival rates comparable to those reported by other centers for similar treatment. In contrast, more extensive treatment has produced the highest survival rates for Stage I-II Hodgkin's disease reported in the world literature. A second major conclusion has been the demonstrated effectiveness of irradiation delivered over a greater than standard period of time, with the result that serious normal tissue reactions and delayed complications have been nearly eliminated despite the extensive nature of the treatment.

Proposed Course: Continued long-term evaluation of surviving patients for evidence of delayed recurrence of disease and/or complications secondary to treatment.

Honors and Awards: None

Publications:

ARTICLES PUBLISHED IN A BOOK

Johnson, R.E.: Modern approaches to the radiotherapy of lymphoma. (In) Seminars in Hematology, Grune & Stratton, New York, 1969.

Johnson, R.E.: The curative radiotherapy of lymphomas. (In) Recent Results in Cancer Research, Springer-Verlag, New York, 1971.

ARTICLES PUBLISHED IN A PERIODICAL

Johnson, R.E., Thomas, L.B., and Chretien, P.: Correlation between clinico-histologic staging and extranodal relapse in Hodgkin's disease. Cancer 25: 1071, 1970.

Johnson, R.E., Thomas, L.B., Schneiderman, M., Glenn, D.W., Faw, F., and Hafermann, M.D.: Preliminary experience with total nodal irradiation in Hodgkin's disease. Radiology 96: 603, 1970.

Curran, R.E., and Johnson, R.E.: Tolerance to chemotherapy after prior irradiation for Hodgkin's disease. Ann. Int. Med. 72: 505, 1970.

Johnson, R.E.: Treatment of Hodgkin's disease with total nodal irradiation, Proc. 10th Intern. Cancer Congress, May, 1970.

Faw, F., Johnson, R.E., Glenn, D.W., and Warren, C.A.: A standard set of "individualized" compensating filters for mantle-field radiotherapy of Hodgkin's disease. Amer. J. Roentgen. 111: 376, 1971.

Johnson, R.E.: Radiation Therapy of Hodgkin's Disease: Total Nodal Irradiation, J.A.M.A. (In Press).

Serial No. NCI-6052 (c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of Prophylactic Chemotherapy in the Management of Ewing's Sarcoma

Previous Serial Number: Same

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Ralph E. Johnson, M.D.

Cooperating Units: None

Man Years:

Total: 1/4
Professional: 1/4

Project Description:

Objectives: To evaluate the usefulness of elective systemic chemotherapy in preventing the development of metastases in patients with clinically localized Ewing's sarcoma.

Methods Employed: The primary tumor site is treated with radiotherapy and systemic chemotherapy is administered in an attempt to eradicate clinically occult metastases.

Major Findings: A total of 26 patients with clinically localized disease have been treated to date with prophylactic chemotherapy. Irradiation of the central nervous system has been added to the treatment program to eradicate possible metastatic disease protected from drug effect by the blood-brain barrier. An increased disease-free survival has already been observed using this approach with approximately 50% of patients surviving without recurrence at two years following initial treatment.

Proposed Course: Accession of patients into this study continues.

Honors and Awards: None

Publications:

Johnson, R.E., Senyszyn, J.J., Rabson, A.S., and Peterson, K.A.: Treatment of Ewing's sarcoma with local irradiation and systemic chemotherapy: a progress report. Radiology 95: 195-197, 1970.

Senyszyn, J.J., Johnson, R.E., and Curran, R.E.: The treatment of metastatic Ewing's sarcoma with D-actinomycin. Cancer Chemo. Rep. 54: 103, 1970

Marsa, G.W., and Johnson, R.E.: Altered pattern of metastasis following treatment of Ewing's sarcoma with radiotherapy and adjuvant chemotheray. Cancer (in press)

Pomeroy, T.C., and Johnson,R.E.: Integrated Therapy for Ewing's sarcoma. Proc. Amer. Assoc. Cancer Res. 12: 80, 1971

Johnson, R.E., and Pomeroy, T.C.: Integrated Therapy for Ewing's sarcoma. Amer. J. Roentgen. (In Press).

Serial No. NCI-6054(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Radiotherapeutic Management of Lymphoproliferative Diseases (Chronic Lymphocytic Leukemia and Lymphocytic Lymphomas)

Previous Serial Number: Same

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: Frederick L. Faw, M.S. and Dwight W. Glenn, M.S.

Cooperating Units: None

Man Years:

Total: 9/6
Professional: 9/6

Project Description:

Objectives: The purpose is to evaluate the clinical staging, diagnostic evaluation, classification, biological behavior, and treatment of patients with lymphoproliferative disorders.

Patient Material: Patients with chronic lymphocytic leukemia, and malignant lymphoma of the lymphocytic type without previous treatment are included in this study.

Methods Employed: All patients are being extensively evaluated with respect to anatomic extent of disease, immunologic competence, and hematologic function and these parameters are serially examined throughout the course of the disease. Treatment techniques being investigated include total body irradiation and extensive systematic irradiation of the lymphatic system.

Major Findings: Approximately 70 patients have been studied to date with the available evidence indicating that major improvements in both remission rates and survival can be achieved with radiation therapy.

Proposed Course: The accession of patients into these studies has been markedly accelerated during the past year and will continue.

Honors and Awards: None

Publications:

ARTICLES PUBLISHED IN A BOOK:

Johnson, R.E.: Discussion of the Treatment for Chronic Lymphocytic Leukemia, (In) Recent Results in Cancer Research, Vol. 36, Publ. Springer-Verlag, New York, 1971.

Johnson, R.E.: The Curative Radiotherapy of Lymphomas, (In) Recent Results in Cancer Research, Vol. 36: Publ. Springer-Verlag, New York, 1971.

ARTICLES PUBLISHED IN A PERIODICAL:

Johnson,R.E.: Total body irradiation of chronic lymphocytic leukemia: Incidence and Duration of remission. Cancer 25: 523-530, 1970.

Johnson, R.E., O'Conor, G.T., and Levin, D.: Primary management of advanced lymphosarcoma with radiotherapy. Cancer 25: 787, 1970.

Serial No. NCI-965(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Radiation Therapy of the Hypothalamic-Pituitary Lesion of Hand-Schuller-Christian Disease

Previous Serial Number: None

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Mortimer Lipsett, M.D., Griff Ross, M.D., and Glenn Braunstein, M.D.

Cooperating Units: National Institute of Child Health and Development - Reproduction Research.

Man Years:

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description:

Objectives: To determine the results of radiation therapy directed to the hypothalamic-pituitary region on the growth hormone production and the severity and progression of diabetes insipidus in patients with Hand-Schuller-Christian disease.

Patient Material: Fifteen patients in the pre-pubertal age range with biopsy proven Hand-Schuller-Christian disease with diabetes insipidus, with or without clinical growth retardation, but without generalized disease will be acceded to the study.

Methods Employed: Extensive clinical and laboratory evaluation of each patient will precede radiation therapy. This will include growth hormone assays and water loading tests. Radiation therapy (1200 rads in two weeks) will be followed by serial determinations of growth hormone production and evaluation of any changes in growth patterns and severity of diabetes insipidus.

Major Findings: Results in the first case treated, though preliminary, have shown an increase in the growth hormone level after radiation, but without increase in rate of growth. Sufficient time has not passed for further determination of results on this and other patients.

Proposed Course: Continue accession of the planned number of patients.

Honors and Awards: None

Publications: None

Serial No. NCI-964(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Use of Systemic Chemotherapy as an Adjuvant to Radiation Therapy

Previous Serial Number: None

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Ayub K. Ommaya, M.D., Frank Wilson, M.D., Gerald Marsa, M.D., Robert K. Oldham, M.D., Ralph E. Johnson, M.D., and Walter G. Hardy.

Cooperating Units: National Institute of Neurological Diseases and Stroke - Surgical Neurology Branch

Man Years

Total:	3/4
Professional:	2/4
Other:	1/4

Project Description:

Objectives: To evaluate the comparative toxicity and usefulness of various chemotherapeutic agents as adjuvants to radiation therapy in patients with malignant tumors of the mouth and throat and Grade III and IV astrocytomas of the brain.

Laboratory investigations with animal tumor systems will attempt to elucidate additive or synergistic responses and/or toxicity of various combinations of modalities and sequences of administration.

Patient Material: Patients with post-surgical and post irradiation recurrences, advanced inoperable squamous cell carcinomas of mouth and throat and primary Grade III and IV Astrocytomas are accepted for study.

Methods Employed: (1) Post-surgical and post radiation recurrences of squamous cell carcinomas of mouth and throat are evaluated for toxicity and tumor response to selected agents for which evidence exists suggesting a response of this type of malignancy (e.g. Bleomycin and Methotrexate). (2) Active drugs and combinations of active drugs will be added to radiation therapy in post-surgical recurrent and advanced inoperable epidermoid cancers of the mouth and throat. (3) Grade III and IV astrocytomas receive Methotrexate infusion and/or perfusion in conjunction with radical radiation therapy. This method

is facilitated by the prior insertion of an Ommaya reservoir. (4) Laboratory studies combine radiation of solid implants with systemic chemotherapy in varied sequences and time-dose relationships.

Major Findings: (1) Limited observations indicate guarded optimism for the usefulness of Bleomycin in management of squamous cell carcinoma. In particular its lack of hematologic toxicity enhances its use with other types of agents. Addition of MTX has shown an apparent synergistic action in two patients with complete clinical disappearance in both and histopathologic disappearance in one. A third patient has also shown a very striking additive effect. Further studies are required to fully document these observations. (2) Adjuvant chemotherapy and radiation therapy of post-surgical and advanced inoperable cases have not been initiated at this time. (3) Excellent tolerance of combined MTX infusion and radical radiation of Grade III and IV astrocytomas has been observed. Long term observation of results is required for determination of efficacy of the treatment. (4) Tumor model systems in mice have shown additive effects of MTX and radiation, Bleomycin and radiation and the best tumor control with MTX, Bleomycin and radiation. Bleomycin combined with hyperbaric oxygen showed a greatly increased pulmonary toxicity indicating the necessity for extreme caution in any clinical trials combining these two modalities.

Proposed Course: To continue and expand these studies.

Honors and Awards: None

Publications: None

Serial No. NCI-6050 (c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Computer Applications in Radiation Therapy

Previous Serial Number: Same

Principal Investigator: Frederick L. Faw, M.S.

Other Investigators: Dwight W. Glenn, M.S., and Robert W. Swain, B.S.

Cooperating Units: None

Man Years:

Total:	12/12
Professional:	12/12

Project Description:

Objectives: To develop techniques for the utilization of computers in increasing the speed and accuracy with which complicated dosimetric calculations can be performed.

Methods Employed: An 'in-house" computer located in the Radiation Branch has been programmed for use in clinical radiation therapy planning. This computer is now "on-line" with the IBM 360 series computer located in the Division of Computer Research and Technology.

Major Findings: Computer programs are now available for routine use in the planning of multiple field external beam radiation therapy and for calculation of dose distributions for radium therapy. The new research linear accelerator is also being evaluated for clinical application by obtaining isodose distribution data through computer control.

Proposed Course: Eventually, it is anticipated that all treatment planning and treatment administration will utilize computer control in the Radiation Branch and plans are currently underway to employ the computer for patient data storage and retrieval purposes.

Honors and Awards: None

Publications: None

Serial No. NCI-961
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biologic Effects of Radiation on Mammalian Tumor Cells

Previous Serial Number: Same

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: Walter G. Hardy

Cooperating Units: None

Man Years:

Total:	6/12
Professional:	1/12
Other:	5/12

Project Description:

Objectives: To study the effects of ionizing radiations on the biologic characteristics of radiation repair in mammalian tumor cells.

Methods Employed: The decreased ability of mammalian cells to repair sublethal damage which reflects itself in increased effectiveness of fractionated irradiation is being observed with high LET radiation compared to low LET radiation. The implication of such findings is related to relative absence of recovery between exposures which is characteristic of currently used treatment modalities and which will require a reassessment of total radiation doses delivered to human tumors if sources for high LET radiations become available for clinical investigation.

Proposed Course: To be completed during the coming year.

Honors and Awards: None

Publications: None

Serial No. NCI-963
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Immunologic Manipulation on Radiation Carcinogenesis and Radiation Response of Tumors

Previous Serial Number: None

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Walter G. Hardy

Cooperating Units: None

Man Years

Total:	4/4
Professional:	1/4
Other:	3/4

Project Description:

Objectives: To study the effects of immunologic manipulation in the incidence and time of appearance of radiation induced tumors and on the radiation response of tumors.

Methods Employed: Total body radiation of mice with cyclic immunosuppression and in vitro radiation of mouse kidney cells with reintroduction of cells intravenously in cyclically immunosuppressed mice are the methods utilized to evaluate the alt erations in types of tumors and time of development of radiation induced benign and malignant neoplasms. Parallel control groups are expected to reveal differences in "spontaneous" tumor production.

Previous studies have shown variations in tumor responsiveness to radiation by non-specific immunologic suppression and enhancement. Presently histopathologic description and correlation of the attendant tissue reactions is being continued.

Major Findings: Preliminary results suggest a delay in appearance of lymphoid tumors in immunosuppressed mice as anticipated. A few adenocarcinomas have developed after in vitro radiation of kidney cells but further efforts will be required to develop this approac h as a satisfactory model using isologous kidney cells rather than pooled kidney cells. Histopathologic changes resemble those of graft rejection where associated with either immunologic enhancement or recovery from immunologic suppression.

Proposed Course: Continue studies with radiation carcinogenesis and add chemical carcinogenesis with a similar model system.

Honors and Awards: None

Publications: None

Serial No. NCI-650 (c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Service Radiation Therapy

Previous Serial Number: Same

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Frederick L. Faw, M.S., Dwight W. Glenn, M.S., Marilyn K. Glover, R.N., and Sandra K. Marshall, R.N.

Cooperating Units: None

Man Years:

Total:	3/2
Professional:	2/2
Other:	1/2

Project Description:

Objectives: To provide expert radiotherapy consultation and treatment for Clinical Center patients admitted to services other than the Radiation Branch of the National Cancer Institute.

Patient Material: During the twelve month period preceding this report, 228 patients were formally seen in consultation. Advice and management of additional patients was provided on an emergency or "ad hoc" basis.

Methods Employed: The majority of patients were irradiated with Cobalt teletherapy following appropriate evaluation and treatment planning as indicated.

Major Findings: Aside from results on research patients as described in project reports from other institutes, the response to radiation therapy has been consistent with that reported from other major centers.

Proposed Course: To continue.

Honors and Awards: None

Publications: None

Serial No. NCI-684
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Non-Clinical Service Irradiation

Previous Serial Number: Same

Principal Investigator: Robert W. Swain

Other Investigators: Thomas L. Bryant and Gilbert M. Bowie

Cooperating Units: None

Man Years:

Total:	4/4
Professional:	2/4
Other:	2/4

Project Description:

Objectives: To provide facilities for the performance of irradiation of biological materials and animals as required for research studies by investigators outside of the Radiation Branch. This includes consultation as to methods and types of irradiation and the preparation of material.

Methods Employed: The three Mev Van de Graaff generator for high energy electrons and x-rays and a 250 Kvcp x-ray generator are provided and maintained in proper operating condition.

Major Findings: The attached tables indicate the organizations for which irradiations were performed and the number of exposures.

Proposed Course: To continue

Honors and Awards: None

Publications: None except as indicated by the use of these facilities in publications from other laboratories.

Services Provided Using the 250 KVCP
Double Header Westinghouse Quadrocondex
from April 1, 1970 to April 1, 1971

Organization	Number of Exposures
A-A&R	117
A-LPB	104
BS-LP	5
BS-LPPB	5
BS-LVI	10
C-BGY	241
C-CCNSC	9
C-CT	8
C-D	14
C-E	10
C-GL&C	52
C-I	242
C-LBGY	112
C-LMP	26
C-LP	2
C-LPHY	529
C-M	257
C-MET	146
C-PA	105
C-R	118
C-SURG	174
C-VB	55
C-VLL	47
CC-CP	2
D-LB	75
C-LM	7
I-LCI	61
I-LI	134
I-LMI	137
I-LVD	335
MH-LCM	29
*MICRO	142
Total	3310

*Exposures for Microbiological Associates were performed on materials for projects under contract to the National Cancer Institute.

Materials irradiated included cells, tissue cultures, blood, mice, rats, hamsters, chickens, and guinea pigs. Exposures ranged from 100 R to 15000 R.

Services Provided Using the
FD Van de Graaff Generator
from April 1, 1970 to April 1, 1971

Organization	Number of Exposures
C-LBGY	5
CC-CP	6
H-LTD	7
I-LI	1
N-PR	4
R-LA	16
Navy	11
Walter Reed	8
	58

Materials irradiated with electrons were animal food, albumin, sucrose, dura heart valves, cells. Total doses from electrons ranged for 10^4 to 10^7 rads.

Serial No. NCI-947
1. Radiation Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development, Fabrication and Maintenance of Specialized Equipment for Radiation Branch, Laboratory of Physiology, and several other Laboratories in NCI

Previous Serial Number: Same

Principal Investigators: Robert W. Swain, Nathan F. Coffey and Edward J. LeBrun

Man Years:

Total:	4.1
Professional:	2.1
Others:	2.0

Project Description:

Objectives: After discussion with the senior scientists in the NCI located in Building 10, designs and fabricates instruments that are not available on the open market. Modifications of existing equipment are also performed to increase its versatility, accuracy, and requirements. In addition, servicing and repairing heavy equipment, such as Van de Graaff generators, x-ray equipment, and mechanized automatic recording devices are undertaken. Glass blowing services, including designing, fabrication, and repair, are also provided.

MACHINE SHOP

The following percentages indicate proportion of major work performed by Machine Shop for various NCI laboratories:

	% OF TIME	HOURS	NO. OF JOBS
C-LPHY	32	986	75
C-R	30	902	47
C-I	12	355	33
C-PA	7	208	13
C-M	6	184	14
C-BGY	6	186	26
C-MET	4	129	11
C-LCBGY	3	93	14
TOTAL		3043	233

Approximately 150 hours consultation with various investigators.

GLASS SHOP

	% OF TIME	HOURS	NO. OF JOBS
C-LPHY	50	1703	105
C-I	21	722	60
C-BGY	25	875	46
C-LPH	3-1/4	106	6
C-SURG	3/4	20	2
	TOTAL	3426	219

Approximately 75 hours consultation with various investigators.

ELECTRONICS SHOP

	% OF TIME	HOURS	NO. OF JOBS
C-R	69		4
C-LPHY	29		29
C-I	1		3
R-BEI	less than 1		1
ODA A PS	less than 1		2
C-EA	less than 1		1
		TOTAL	40

ANNUAL REPORT SUMMARY

SURGERY BRANCH

NATIONAL CANCER INSTITUTE

April 1, 1970 - March 31, 1971

The Surgery Branch has continued their dual responsibilities of providing surgical consultative and operative assistance to the multi-disciplined intramural clinical units of the National Institutes of Health while developing its own laboratory and clinical research programs. Approximately 60% of the Surgery Branch staff's activities are consumed by clinical responsibilities. During the past 12 months, 655 individually submitted surgical consultations were received in the Branch office in addition to supervising 794 requests for consultative services from extramural consultant sources. These figures include only the original consultative requests and are not inclusive of the tremendously larger total number of visits that were repetitively made to follow up the surgical consultative recommendations or surgery itself. Surgical procedures were performed upon 804 patients during the past year, of which only 300 were performed as part of the specific clinical research program of the Surgery Branch itself.

The long-term clinical program investigating the effects of aggressive surgery on carcinoma of the cervix is gradually being phased down. Patients with radiation recurrent cancer of the cervix or advanced Stage III and Stage IV lesions have been shown to have a 36% five-year survival following pelvic exenteration. This is a uniquely high figure in comparison to the 0-5% survival by other modes of therapy. Accompanying this 168 exenterative procedures is 84 patients who underwent radical hysterectomy and pelvic lymphadnectomy. The overall five-year survival in this group of patients is 86.5%, the bulk of whom were Stage II and several of Stage III lesions.

The continued interest in paranasal sinus cancer is satisfied by the 60% survival now obtained in patients who underwent the NIH developed cranial facial exenteration for disease which involves the ethmoid sinuses. The use of preoperative radiation as an adjuvant to disease of this and other anatomical areas of head and neck has not proven to be of value by increasing survival but has been of extreme significance in increasing morbidity. A cooperative adjuvant chemotherapy program is now underway in an attempt to offer further therapeutic modalities to patients with advanced head and neck cancer.

The other two active clinical programs involve around patients with malignant melanoma and soft tissue and bone sarcomas. These two particular disease entities have recently become a part of the Branch immunotherapy studies.

Immunotherapy of Human Melanoma

This treatment took two forms: 1) The administration of intradermal autologous cellular vaccines; 2) The administration of BCG vaccine directly into cutaneous melanoma nodules. The disease, aside from being far advanced, was

situated in such a way as to make it possible to render the patients clinically tumor free. All patients were determined to be immunologically competent according to their reactions to common skin test antigens as well as their ability to become sensitized to cutaneously applied DNCB.

Of the 14 patients who received cellular vaccination therapy, one patient is disease free 9 months after initiation of therapy. The remaining 13 patients all had recurrences of their disease, and are estimated to have no alteration of their clinical status due to cellular vaccination therapy. Although the assay for humoral cytotoxic antibody has not been perfected with melanoma tissue culture cell lines, initial results would indicate that no alteration of cytotoxic antibody was noticed as a result of administration of cellular therapy.

Of the 18 patients who received BCG injections to cutaneous nodules all had regression of at least several nodules as a result of direct BCG innoculation. However, in 16 of these 18 patients no alteration of their clinical courses was noted. Two patients of the 18 who received BCG injections into cutaneous nodules are alive and without clinical disease at 3 1/2 years and one year following initiation of therapy. In both of these patients, it is to be noted that their cutaneous metastases are in the form of in transit metastases and that no visceral metastases were present at any time. Both of these patients have been demonstrated to have large amounts of cytotoxic antibody in their sera.

Forty-seven patients have now undergone hemipelvectomies for soft tissue and bone sarcomas of the lower extremity. The survival in this group of patients is most satisfactory if the osteogenic sarcomas are eliminated from this series. We have been extremely disappointed with the results of the immunotherapy studies in the group of patients with recurrent sarcoma. 14 patients have received radiated autologous tumor cells mixed with BCG by multiple intradermal injections. 14 of these intradermal cellular vaccinated patients had recurrence within 5 months and there has been no observed clinical alteration in the progressive nature of the malignant disease process.

In several animal tumor systems, it has been repeatedly demonstrated that a safe level of constant coumadin anticoagulation can be attained through heparin medicated drinking water. Using spontaneously arising and intramuscularly transplanted tumor, anticoagulated animals have a significant reduction in the number who develop metastases and in the amount of metastatic disease occurring in those animals who do develop metastases. The results of this work have now been incorporated in a clinical protocol in which patients undergoing surgery for sarcoma, known to be prone to hematogenous tumor dissemination, are anticoagulated preoperatively and postoperatively maintained indefinitely with a two to three times increase in their prothrombin time. In the 7 patients who have thus far been entered into this protocol and maintained for six months on anticoagulation there has been some increased operative morbidity, but of a nature not significant to interfere with the continuation of the protocol investigation.

One of our laboratories is investigating the properties of preparations rich in ribonucleic acid extracted from sites of antigen processing (such as the lymph nodes and spleen, or peritoneal macrophages) of animals immunized with tumors in mediating immune responses to transplantation and to tumor-specific antigens. We have investigated this phenomenon in vivo and in vitro utilizing heterologous as well as homologous and isologous sources of RNA. Noted is the inhibition of the growth of tumor isografts in inbred mice by the administration of syngeneic spleen cells which had been incubated prior to injection with RNA extracted from the lymph nodes and spleens of guinea pigs immunized with the same tumor used to challenge the RNA-incubated spleen cell recipient. In two completely syngeneic systems, we have mediated immune cytolysis of tumor cells with "immune RNA" directed against tumor specific transplantation antigens. When monolayers of a methylcholanthrene-induced liposarcoma of inbred Strain 2 guinea pigs were exposed to syngeneic guinea pigs hyperimmunized to the Strain 2 guinea pig liposarcoma, zones of immune cytolysis were produced. In another syngeneic system, the growth of isografts of a benzpyrene-induced sarcoma in inbred rats has been inhibited by the intravenous administration of syngeneic spleen cells which have previously been incubated with RNA extracted from the spleens of syngeneic rats hyperimmunized with the same tumor. Recently, we have inhibited the growth of tumor isografts in inbred rats by injecting "immune RNA" itself directly into the rats. This RNA is mixed with dextran sulfate, sodium salt, molecular weight 77,000, prior to injection into the foot pads of the rats.

Previous data from this Branch have demonstrated that there is common antigenicity among some of the mouse mammary tumor viruses as measured by in vitro techniques. During this past year, data have been obtained on in vivo demonstrations of a similar cross-reactivity. Animals immunized against either the R3 virus or the C3H virus have then been subsequently challenged with syngeneic tumor due to the same viruses. Immunologic reactivity is demonstrable against both R3 virus-induced tumor and C3H virus-induced tumor whether the animals were previously sensitized with tumor due to the same virus or tumor due to the other virus. The inescapable conclusion is that there is common antigenicity between at least two strains of mouse mammary tumor virus. By analogy with human systems, this suggests the possibility of common antigenicity among human breast cancers.

Studies of lymphocyte stimulation in patients with cancer has demonstrated a wide range of lymphocyte reactivity, but the reactivity in patients with advanced head and neck and cervix malignancies is usually subnormal. These patients will be followed after definitive treatment of their malignancy by surgery in the hopes of demonstrating a correlation with their clinical fate with their preoperative lymphocyte reactivity. Moreover, a suppressant of lymphocyte reactivity has been detected in the sera of certain cancer patients. Recently, we have demonstrated increased reactivity of lymphocytes from sarcoma patients on exposure to an extract of human sarcoma cells. This represents the first demonstration of an in vitro cellular reaction to human sarcoma antigens.

Immunologic and virus studies with human sarcomas conducted by the Tumor Immunology Section have resulted in major scientific advances that have

attracted worldwide attention. A complement fixation test for the study of a human tumor antigens and antibodies has been developed which utilizes selected fresh human serum as a complement source. Using this test, sarcoma specific antibodies have been found in 92% of the sera from patients with skeletal and soft tissue sarcomas with a mean titer of 1/98. The incidence of antibody in the serum of normal blood bank donors is 21% with a mean titer of 1/12. Sera from patients with other types of malignancies were found not to contain a higher incidence or titer of antibody than normal blood bank donors. The titer of this antisarcoma antibody shows an excellent correlation with the clinical course of a patient's malignant disease. A common sarcoma specific antigen has been found in tissue culture cells derived from several histologic types of human sarcomas. This sarcoma specific antigen is not present on normal fibroblasts of sarcoma patients, in nonsarcomatous malignancies, normal human organs or fetal organs. The sarcoma specific antigen is cell associated but it can be solubilized from tissue culture cells of human sarcomas by a variety of techniques. This soluble sarcoma antigen is quite stable and has been partially purified by chromatogoaphy and polyacrylamide gel electrophoresis. This antigen appears to be a protein since it is partially inactivated by trypsin and completely inactivated by pronase. The partially purified antigen reacts well with specific antisarcomas but not in patients with other types of malignancies. Hopefully, isolation and characterization of this sarcoma specific antigen will provide an immunogen for the immunotherapy of human sarcomas. The possible relationship of this sarcoma specific antigen to an associated viral agent in human sarcomas has been suggested by the following evidence: 1) There is a common cross-reacting sarcoma specific antigen found in all different histologic types of skeletal and soft tissue sarcomas. Since all animal neoplasms induced by the same virus contain a common viral specific tumor antigen, the presence of a common antigen in human sarcomas suggests a viral etiology. 2) The serum of 79% of the immediate family members of sarcoma patients has been found to contain antisarcoma antibody to a mean titer of 1/67, which is significantly higher than the antibodies of antibody titer and incidence of the normal blood bank donors. This suggested the presence of an agent in human sarcomas that is capable of infecting associates of sarcoma patients. 3) The presence of a filterable agent in human sarcomas was directly demonstrated by the finding that cell-free filtered extracts both from sarcoma tumor tissue and from concentrated media from tissue cultures of human sarcomas were capable of infecting normal human embryonic cells in tissue culture where they induced the formation of a new sarcoma specific antigen. These antigenically transformed normal cells reacted with serum from sarcoma patients whereas normal uninfected cells did not. Absorption of the sarcoma test sera with the sarcoma tissue culture cells removed all the activity against the original sarcoma culture as well as against the transferred antigen. Indicating that the filterable agent inducing the new antigen was the same as the agent inducing the antigen in the original sarcoma. 4) Virus particles morphologically similar to the "C" type sarcoma viruses of the avian, murine, and feline species have been detected in a human liposarcoma. Cytotoxic antibody directed against a common cross-reacting sarcoma antigen has been detected in 50% of sarcoma patients when tested in vitro against sarcoma cells. If this sarcoma specific antigen can be isolated and purified, it might be a powerful immunogen for use in active immunotherapy. Also, it is possible

Specific anti-melanoma antibody has been found by complement fixation in the sera of melanoma patients against a common melanoma antigen derived from tissue cultures of human melanomas. This antigen is not found in normal fibroblasts, other malignancies, normal adult or fetal human cells in tissue culture. Anti-melanoma antibody was present in higher incidence and titer in patients with localized melanoma than in patients with advanced disease. Preliminary studies in immunotherapy of malignant melanoma using BCG vaccine were undertaken in eight patients with diffuse subcutaneous metastases. Metastatic lesions directly injected with BCG. Some nodules were left uninjected for control observations. Five of the eight patients have shown some response to this therapy with regression of over 90% of the nodules directly with BCG. Two patients have also exhibited regression of uninjected nodules.

Our original observations demonstrating a correlation between the ability to display delayed cutaneous hypersensitivity to DNCB and prognosis following definitive cancer surgery, has been extended. This correlation appears correct for squamous cell carcinoma of the head and neck, uterine cervix and malignant melanoma where 39 of 40 patients or 97% of patients who could not be sensitized to DNCB were found to be inoperable or developed recurrence within six months of surgery. However, sarcoma patients are capable of manifesting delayed cutaneous hypersensitivity to DNCB even in the presence of widespread disease.

Soluble tumor specific transplantation antigens have been extracted from guinea pig sarcomas which are capable of inducing transplantation resistance to the immunizing tumor and eliciting delayed cutaneous hypersensitivity. These antigens have been partially purified by chromatography and gel electrophoresis. We have also found that intradermal immunization with live sarcoma cells will completely prevent the development of tumor growth following inoculation of the same tumor at an intramuscular site.

Cellular immune responses to tumor specific transplantation antigens have been evaluated by a new assay for immune cytolysis using I^{125} iododeoxyuridine labeled target cells. This technique demonstrates the development of tumor specific cellular immunity in animals. Studies of cellular immunity in several animal tumor host systems are in progress. Human patients with malignant melanoma, sarcoma, and lung cancer are also being studied for cellular immunity to tumors.

The phenomenon of preferential metastasis as vividly demonstrated by Kinsey was used to explore the autogenous biologic potential of viable tumor cells shed from pulmonary metastases. This study has direct bearing on the question, Do metastases metastasize? and also has major implications for the immunity of the host to tumor cells circulating in the blood stream. First, preferential metastasis was demonstrated in the fifth generation of a methylcholanthrene-induced sarcoma of C57 mice in syngeneic C57 black mice. Heterotopically transplanted organ fragments of newborn syngeneic C57 mouse lung, kidney and liver were aseptically placed subcutaneously. Forty-eight hours before transplantation of these organs the animals received a transplant of 10^6 tumor cells in the leg. Spontaneous metastases occurred only to pulmonary tissue, whether it be the animals own tissue or the transplant. Other organs and other transplants never developed tumor. In a second group of experiments, transplants were placed after amputation of a large leg primary, amputation having been performed at a stage where pulmonary metastases develop in the pulmonary transplant, and all the pulmonary metastases in the host were of approximately the same size. These data suggest that pulmonary metastases themselves are not capable of re-metastasizing to sites of preferential metastasis. In a group of untreated animals, the left heart blood was removed and inoculated into syngeneic recipients. In eight of eight such bioassay and recipients, tumor cells shed from the pulmonary metastases in left heart blood. It was concluded that within the context of this tumor host system, which is a highly immunogenic tumor host system, that metastases do not metastasize. The reasonable explanation for this finding was that tumor specific immunity, perhaps facilitated by removal of the primary lesion, prevented growth in the transplants which were previously sites of preferential metastases.

A study of the effect of route of tumor cell inoculation showed that, in 2 of 3 mouse tumor systems, more animals developed pulmonary tumors after I.V. inoculation than did the animals develop leg tumors at the site of I.M. inoculation. Because of the potential relevance of this phenomenon to the development of metastases from circulating tumor cells in humans with malignancies, a systemic investigation of the factors involved in this differential host-tumor relationship in different anatomical areas is in progress. Thus, these investigations have been conducted with an MCA tumor induced in C57B1 6N mice. The studies on the effect of prior immunization to the tumor show that blood containing circulating tumor cells spontaneously shed from an MCA tumor produces the same incidence of pulmonary tumors in immune and non-immune mice. The same results were obtained with large numbers of tumor cells (10^3) injected IV.

Small tumor cell doses (500, 100, 50) inoculated IV, however, produced progressively fewer pulmonary tumors in control mice than in immune mice, and the tumor incidence in both was markedly higher than in mice, immune and control, receiving the same number of tumor cells I.M. Splenectomy decreased the incidence of pulmonary tumors after IV inoculation of tumor cells in both control and immune mice. The increased survival and proliferation of I.V. administered tumor cells in mice that are immune to tumor (as demonstrated by resistance to growth of IM inoculation) and the reduction of this effect of immunization by splenectomy point to immunological mechanisms that protect IV tumor cells. Further investigations will attempt to define this mechanism

and the relevance to the development of blood borne metastases in humans with cancer.

Using the techniques of the molecular biologist in investigations of genitourinary disease, changes in the subcellular environment of renal cells following acute ureteral obstruction and contralateral ureteral ligation have been outlined. Following acute ureteral ligation, there is an immediate increase in bulk RNA, protein and DNA in the mouse kidney. However, after three days, accretion of RNA and protein cease but DNA synthesis apparently continues. The inability of the cell to continue to make RNA suggests an early defect in transcription. Later, protein synthesis fails, at least in part, because of a disruption in the polyribosomes. Thus, there appears to be an initial but unsustained attempt at growth in the obstructed kidney. The stimulus for this growth may be the same as that which initiates compensatory hypertrophy in the contralateral mate.

The techniques of molecular biology have also been applied to the study of tissues transformed by DNA oncogenic viruses. The organization of cytoplasmic RNA has been described. Major findings were: (1) all transformed tissues had less than 10% membrane-bound ribosomal RNA, and (2) polyribosome profiles in all tumors were characterized by a lack of heavy, rapidly-sedimenting polysomes and preponderance of oligosomes dominated by prominent monomer and dimer peaks. Studies are continuing to determine whether the distribution of polyribosomes within these tumors is characteristic; i.e., is this a so-called "molecular fingerprint" of these viruses.

Three morphologically distinct cell lines of adenomatous prostatic tissues have been grown in vitro. One cell line has now been carried through 1-0 subcultures. The criteria to identify the prostatic epithelial origin of these cells have been established. The intracellular "prostatic" acid phosphatase was found to be high and these cells take up large quantities of zinc in response to testosterone in vitro. At the fine structure level, the cells show desmosomes, a well-developed rough endoplasmic reticulum and many microvilli -- findings consistent with their being an epithelial cell with secretory capacity.

This laboratory is engated in physiological studies in patients and animals after major surgical trauma. Four main areas of investigation are under study and include the following: The study of fluid volume and albumin kinetics; capillary permeability studies in states of stress and other trauma; studies on etiology, pathogenesis, and treatment of the syndrome post-traumatic pulmonary insufficiency (congestive atelectasis); and the development of a method of auto-transfusion to be used during extensive cancer surgical procedures.

Significant developments to date include the following: After major cancer surgery we have demonstrated a loss of 24% of the patient's plasma volume into an non-circulating or nonavailable pool. In addition some 33% of the total circulating proteins disappears from the circulation during the course of a major surgical procedure. This protein loss from the circulation is not available to the body as in metabolic measurements of the total body albumin

before and after major surgery up to 29% of the total body albumin is in a non-exchangeable area. This sequestration of albumin into a non-circulating or non-exchangeable pool remains in this area for a period of 7-10 days. We were unable to demonstrate a rapid influx of albumin from the extra vascular compartment into the vascular system after a major surgical procedure and indeed the loss of albumin from the vascular pool remained in between 20 and 30% deficient for a period of at least 24 hours postoperatively. These studies indicated the need for vigorous replacement albumin during and following a major surgical procedure and indeed we have found that as much as 300 grams must be given to some patients over a 24 hour period. Since the institution of this regimen of management abnormalities in the patient's physiological functions postoperatively have been much less frequently observed.

For the study of capillary permeability we have completed standardization of gel columns and automatic determination of dextran fractions. We now have the capability to study changes in capillary pore size varying from 10,000 to 150,000 molecular weight.

In the experimental animal we have reproduced a pathological picture of post traumatic pulmonary insufficiency in an isolated perfusion lung circuit. Studies are currently underway to evaluate the various causes of factors believed to be associated with this uniformly fatal postoperative complication. Heretofore, the syndrome has been difficult to reproduce experimentally and the lack of a suitable model has interfered with progress in understanding the disease.

In conjunction with bio-medical engineers suction device has been developed which will permit the surgeon to anticoagulate blood as it is suctioned from the operative area at the time of surgery. The blood is then filtered through a pump system and returned to the same animal to avoid necessity for homologous blood transfusion to replace that commonly lost at surgery. Hemologsis is minimal even with the turn over of up to 20 times the animal's blood volume. Clinical trials of the suction device and system will currently be in progress.

In addition this laboratory in conjunction with the Department of Cytopathology has instituted a program to evaluate the effectiveness of find needle (gauge 22) aspiration biopsy in the cytologic diagnosis of solid tumors. This technique has been proven to be of extreme value in the Scandinavian countries, especially in early diagnosis of breast, prostate, thyroid and salivary gland cancer. It has failed to gain momentum in this country because of the technical difficulties experienced with making the smear and interpretation of the slides using the hematology stain. A cooperative study is now in progress to evaluate the technique using several different kinds of cytological and hematological stains and in a preliminary trial period the results have indeed been encouraging. All lesions thus far have proven histologically to show cancer was diagnosed preoperatively as cancer using this technique by either employment of the Papanicolaou or hematology stain.

TABLE 1

Individual Written Intramural Consultations Received by Surgery Branch, NCI
Listed by Institutes
1963-1970

	NCI	NINDS	NIAMD	NHLI	NIAID	NIMH	NIDR	EHS	TOTAL
1963	366	184	221	228	158	37	26	-	1220
1964	356	121	207	201	104	33	24	-	1046
1965	429	129	216	277	119	39	31	-	1240
1966	362	271	201	197	86	59	11	7	1205
1967	343	215	245	166	85	61	17	-	1132
1968	278	143	192	203	111	54	25	7	1013
1969	314	131	248	207	128	45	31	10	1114
1970	199	59	137	104	96	41	12	7	655

TABLE 2

Summary of Written Extramural Consultations Requested by or Through
Surgery Branch, NCI
1963 - 1970

	ENT	General Surgery	Gynecology	Urology	Oncology	Total
1963	346	14	7	18	9	394
1964	233	6	4	4	3	242
1965	268	6	2	9	1	286
1966	271	8	4	13	2	298
1967	294	11	1	1	1	308
1968	306	8	1	1	1	317
1969	329	8	2	3	2	344
1970	778*	12**	2	2	0	794

* 52% of these consultations were at the request of Dr. Robert I. Henkin, NHLI,ET, part of his clinical research program.

** Plastic surgery, 10
Thoracic surgery, 2

TABLE 3

Annual Report of NIH Surgical Suites
Listing the Number of Major & Minor Procedures
Carried Out by Each of the Operative Units at NIH
April 1, 1970 - March 31, 1971

Patient	NCI	NHLI	NINDS Neuro.	NINDS Muscle	NIAID	NIAMD	NEI	NIMH	NICHD	NIDR	Total #Pts. Operated		Cons.
Major	279	3	2	0	0	0	1	0	3	2	295		5
NCI												628	
Minor	295	1	2	0	0	0	0	0	8	2	333		25
Major	30	257	0	0	0	0	0	0	0	1	288		0
NHLI												390	
Minor	52	40	0	4	0	0	0	0	2	0	102		4
Major	7	2	104	0	0	0	4	0	0	1	119		1
NINDS												346	
Minor	10	0	14	198	0	1	1	0	1	2	227		0
Major	46	6	3	0	0	0	1	0	2	0	63		5
NIAMD												103	
Minor	24	5	0	5	0	1	0	0	0	0	40		5
Major	16	3	3	0	0	0	0	0	0	0	25		3
NIAID												61	
Minor	23	5	2	4	0	0	0	0	0	1	36		1
Major	0	0	0	0	0	0	0	0	0	3	3		0
NIDR												5	
Minor	2	0	0	0	0	0	0	0	0	0	2		0
Major	2	1	0	0	0	0	0	0	0	0	4		1
NIMH												5	
Minor	1	0	0	0	0	0	0	0	0	0	1		0
Major	2	0	0	0	0	0	0	0	4	0	6		0
NICHD												24	
Minor	9	0	0	0	0	0	1	0	8	0	18		0
Major	4	0	0	0	0	0	30	0	0	0	34		0
NEI												76	
Minor	2	0	0	4	0	0	36	0	0	0	42		0
GRAND TOTAL	804	323	130	215	0	2	74	0	28	12	1638	1638	50

TABLE 4

Number of Surgical Procedures Performed
By NCI Surgery Branch Staff
and Magnitude of the Procedures in 1970

	10 East	Other NCI	Other Institutes	Total
1963	470	159	399	1028
1964	424	215	256	895
1965	394	112	199	705
1966	410	113	218	741
1967	440	145	140	725
1968	394	205	113	712
1969	339	233	158	730
1970	301	273	230	804
Major	202	101	83	386
Minor	99	172	147	418

Serial No. NCI-3800

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Removal of Cancer and Other Tissues for Histologic Biochemical and Other Studies as Required by Scientists and Other Investigators for Correlated Studies

Previous Serial Number: Same

Principal Investigators: Entire Staff of the Surgery Branch, NCI

Other Investigators: Several Branches of the Cancer Institute and many members of other Institutes with whom cooperative studies develop particularly G.D. Aurbach, M.D., P.P. Carbone, M.D., V.T. DeVita, M.D., E.S. Henderson, M.D., K. Engelman, M.D., R.I. Henkin, M.D., R.E. Johnson, M.D., and F.C. Bartter, M.D., L.B. Thomas, R.A. Malmgren, J. Zajicek and J. Linsk, M.D.

Cooperating Units: Anesthesiology Department, Clinical Center and all clinical units of the National Institutes of Health. Cytopathology Department, Karolinska Institute, Stockholm, Sweden and Cytopathology Department, Atlantic City Hospital, Atlantic City, New Jersey

Man Years: (July 1, 1970 through June 30, 1971)

Total:	6½
Professional:	4
Other:	2½

Project Description:

Part I. Service aspects

Part II. Specific Cooperative Studies

A. Pheochromocytoma
B. Hyperparathyroidism
C. Lymphoma and Hodgkin's Disease
D. Mechanism of taste and smell
E. Splenectomy and its role in chronic myelogenous leukemia
F. Fine needle aspiration biopsy in the Diagnosis of solid tumors with cytologic techniques

Project Description:

Part I. Service aspects

Objectives:

The object of this project is to make available human tissue for histologic, histochemical, biochemical, biophysical, viral and immunological studies to scientists working in various branches of the National Cancer Institute and other institutes, who require tissue for studies. As a rule, such studies are not done as cooperative projects. In addition, it implies furnishing general surgical consultative coverage of patients admitted to other branches of the National Cancer Institute and/or other institutes and assisting with their study projects. This surgery is often definitive in nature, the therapy regime requiring surgical tissue excision as the sole type of therapy or more often as an adjuvant to other modes of treatment.

Methods Employed:

Methods employed are standard surgical procedures. Material from this project is obtained mostly from patients hospitalized for study in other branches or institutes. Occasionally it is necessary to admit patients for specific studies in which a specific type of tissue is obtained. Tumor tissue, lymph nodes, muscle, skin and liver and spleen comprise the greater share of tissue removed.

Major Findings:

In that the major portion of the work involved in this project is done as a surgical consultative service and not as a cooperative study, the project results are not available to the surgical service. During the course of supplying this service, 655 individual requests for consultation were received. These figures do not include the extensive follow-up which is afforded each of these consultations but only the original written request to participate in their care. Of these requests 503 resulted in an operative procedure, 37% of which entailed major surgery. See attached Tables 1-4.

Significance to Bio-medical Research and Program of the Institute:

This project is a service function for other branches of the National Cancer Institute and National Institutes of Health. It is seldom that the Surgery Branch is included in the research studies other than supplying the tissue to the investigator, however, this trend is being reversed and several studies are being carried out on a cooperative basis with Surgery Branch Staff now serving as active counselors in the overall study.

Proposed Course of Project:

Continued cooperative assistance within the physical limits of the Surgery

Branch Staff.

Project Description:

Part II. Specific cooperative studies:

A. Pheochromocytoma

Objectives:

The goals of this project have been: 1) the development and evaluation of a joint medico-surgical program for the overall management of patients with pheochromocytoma and 2) continuing clinico-pathologic scrutiny of the operative material from these cases in an effort to define more clearly the malignant potential possessed by this unusual tumor which lacks the usual histologic criteria for malignancy in spite of demonstrating its biologically malignant nature in about 10% of cases.

Methods Employed:

Standard surgical and anesthetic procedures are used when operating upon these patients. The analytical methods for catecholamine determinations are those developed and currently employed by the Experimental Therapeutics Branch, NHLI. The patients studied are those who have been referred to the Experimental Therapeutics Branch, NHLI, for evaluation and treatment of their pheochromocytoma. Both conventional light and electron microscopy are applied to the study of the resected materials. Attempts are underway to grow these tumors in tissue culture in order to develop a means for assessing biologic malignant potential by an *in vitro* procedure.

Major Findings:

Our experience now includes 29 cases and previous conclusions with respect to management of these patients have been reaffirmed. Intraoperative localization of tumor by observation of blood pressure rise in response to manipulation remains satisfactory. Thorough preoperative biochemical preparation of these patients continues to procide a much safer anesthetic and operative course. More data has accumulated to strengthen our concept that total adrenalectomy should be done in all instances of adrenal pheochromocytoma rather than attempt to shell tumor out of a gland which is to be left behind. Preliminary results with tissue culture of pheochromocytoma material suggest that a clinical and histologically benign pheochromocytoma currently cannot be developed into a serially transferable tissue culture line. Further experience with a more malignant lesion is necessary.

Significance to Bio-medical Research and Program of the Institute:

There are few tumors which have a dual uniqueness similar to pheochromocytoma, i.e., a functional lesion with an easily measured secretory produce but which

lacks intrinsic histologic criteria of malignancy. This combination provides a rare opportunity to study the effectiveness of vigorous surgical therapy using as an endpoint something other than the ultimate demise of the patient from disseminated disease.

The demonstration that these tumors will grow for a time in tissue culture, although their transferability into a permanent line has not been achieved as yet, suggests that this means may be useful in assaying degree of malignant potential prior to the development of lethal biological evidence in the patient. The measurement of catecholamines in the tissue culture supernatant and in the cells themselves offers further potential of evaluating efficacy of agents that might have specific anti-tumor activity.

Proposed Course of Project:

The cooperative study has now concluded and the review that is under way will have emphasis placed upon elucidation of relative degrees of malignancy among the various lesions and upon attempts to benefit more patients with malignant pheochromocytomas by meticulous surgery.

Project Description:

Part II. Specific cooperative studies

B. Hyperparathyroidism

Objectives:

The Clinical Endocrinology Branch, NHLI, the Section on Mineral Metabolism, Metabolic Diseases Branch, NIAMD, and the Diagnostic Radiology Branch, Clinical Center, have programs underway to determine clinical, laboratory, and radiographic criteria which justify surgical exploration of the neck for the treatment of parathyroid disease. This joint program is expected to increase the reliability of diagnosis and efficacy of treatment for parathyroid disorders, particularly in early cases where the treatment will reduce the incidence of secondary renal disease.

Methods Employed:

Standard surgical and anesthetic procedures are used for operating on the patients. In addition to the customary extensive clinical evaluation and laboratory determination of static derangements of calcium and phosphorus metabolism, a battery of tests are performed to measure responses to induced distortions of calcium and phosphorus hemostasis. Additionally, selective inferior thyroid arteriographic studies are done preoperatively, as is selective venography and removal of blood samples from various veins draining the parathyroid complex identified by venography. Radioimmunoassay determinations of parathyroid hormone are made upon the venous samples so obtained. At operation, prior to any extirpative procedure, intensive efforts are made

to identify positively by frozen section all parathyroids present.

Major Findings:

Selective inferior thyroid arteriography continues to be of considerable value in the preoperative localization of abnormal parathyroid glands, particularly when the subtraction technique is applied to the arteriograms. Selective venography has not been helpful in finding abnormal parathyroid glands, but has enabled the radiographer to identify the appropriate veins from which to withdraw blood for parathyroid hormone assays. The parathyroid hormone assay data is very encouraging, although as yet only a small number of studies have been done because of the difficult nature of this assay.

In several patients hormone values in the blood draining that area of the parathyroid complex where the abnormal parathyroid was found at operation have been significantly elevated, whereas parathyroid hormone levels from the rest of the venous drainage of this area have been normal.

Long term followup studies eight to eleven years after exploration and removal of abnormal parathyroid tissue from chromic renal stoge formers with normocalcemic hyperparathyroidism have provided support for the concept that early operation is advantageous for decreasing stone formation and preventing subsequent loss of functional renal mass.

Significance to Bio-medical Research and Program of the Institute:

Early diagnosis and removal of over-active parathyroid tissue is an important step toward preservation of renal function from the ravages of stone formation. The addition of selective venous catheterization and radio-immunoassay for parathyroid hormonal levels of samples thereby obtained is an important tool for both diagnosis of primary hyperparathyroidism and preoperative localization of the abnormal tissue. Selected angiography has continued to bear out its earlier promise as an important aid to the surgeon in finding the abnormal glands readily, with a resultant decrease in operating time and morbidity.

Proposed Course of Project:

Continued cooperative studies.

Project Description:

Part II. Specific cooperative studies

C. Lymphoma and Hodgkin's Disease

Objectives:

To assess the adequacy of staging of patients with lymphoma and Hodgkin's

disease by nonsurgical methods. To assess the effect of splenectomy on patient survival and tolerance of radiotherapy and chemotherapy treatment regimens.

Methods Employed:

All patients admitted to the Chemotherapy and Radiotherapy Services with lymphoma and Hodgkin's disease undergo routine staging by the combined evaluation of clinical and physical findings, liver chemistries, lymphangiograms, and chest x-ray. To assess the accuracy of these staging techniques, selected patients have been subjected to laporotomy and biopsy of the liver and abdominal lymph nodes. Selected adenopathy detected after treatment is further evaluated by biopsy and histologic examination.

Major Findings:

The mounting experience gained through the examination of tissues removed at laporotomy in patients with lymphoma and Hodgkin's disease in comparison with other staging data will more accurately delineate those patients who will profit from the inconvenience of laporotomy. Sufficient evidence has accumulated to allow the conclusion that all patients will not benefit from staging by laporotomy, a conclusion that is at varience with the findings of several other major treatment centers. On the other hand, biopsy of multiple abdominal nodal areas has demonstrated the limitations of lymphangiography in assessing the extent of abdominal nodal disease. The contribution made by abdominal laporotomy and biopsy, however, is clearly dependent on the treatment proposed and how it is altered by the finding of unsuspected positive abdominal lymph nodes.

The evidence emerging from other treatment centers that splenectomy favorably affects patient survival and tolerance of radiotherapy and chemotherapy in Hodgkin's disease and lymphosarcoma is currently being assessed at this institution by a prospective study of the effects of splenectomy on these parameters. Although the value of splenectomy in those patients with abnormal consumption of blood cells is continuously being demonstrated as is the laboratory benefit of splenectomy and the salutory benefit of removal of spleens unresponsive to chemotherapy and radiotherapy, sufficient data has not yet accumulated to allow assessment of the effects of splenectomy on tolerance of subsequent specific treatment and overall survival.

Significance to Bio-medical Research and Program of Institute:

The cooperation of the Surgery Branch with the divisions of the Institute concerned with treatment of patients with lymphoma and Hodgkin's disease results in a continuously improving program of diagnosis and treatment of these patients. These studies allow the intelligent selection of those patients who would benefit by laporotomy prior to specific treatment of the disorder.

Project Description:

Part II. Specific cooperative studies

D. Mechanism of taste and smell

Objectives:

To investigate the mechanism of taste and smell and the compensatory alteration in these functions following radical head and neck surgery.

Methods Employed:

Subjective reports of olfactory acuity were obtained in 35 patients who had undergone laryngectomy for carcinoma of the larynx. Each was questioned about his ability to smell before and after surgical removal of the larynx. Detection and recognition thresholds for the vapors of pyridine, thiophene, nitrobenzene, amyl acetate, and camphor were determined objectively in 18 of these patients. Eight were studied both before and after laryngectomy, the others only after operation. Olfactory thresholds were determined by a slight modification of a forced choice-three stimulus sniff technique.

A detection threshold was defined as the lowest concentration of solute and solvent that the patient could consistently discriminate from two solutions of solvent alone. A recognition threshold was defined as the lowest concentration of solute and solvent that the patient could consistently identify correctly as the solute. All thresholds were determined in the afternoon in a room from which extraneous odors had been excluded. The patients had not eaten or smoked for at least an hour before the examination. Subjective reports of olfactory acuity and olfactory thresholds were obtained in some patients as soon after operation as they were alert enough to make meaningful judgements; in some patients this was within two hours after operation.

Subjective reports and detection and recognition thresholds were also determined in three patients before and after tracheostomy. One of these patients has a cuffed tracheostomy tube put in place. Thresholds in this patient were obtained with the cuff both inflated and deflated. Two of these patients subsequently underwent laryngectomy, and subjective reports of olfactory acuity and olfactory thresholds were obtained after this procedure also.

Detection and recognition thresholds for representatives of each of the four taste modalities were also obtained in each of the patients in whom these measurements could be obtained.

Major Findings:

After laryngectomy, all 35 patients studied reported that they had lost their ability to smell. They reported that they were aware of this loss as soon after operation as they were alert; none was able to detect any odor either

from the hospital ward or from their own excrement. Objectively, immediately after operation each patient tested could neither detect nor recognize any difference between water and pure pyridine, 40% formaline, or 10N hydrochloric acid by nasal inhalation. Within two weeks after operation some could distinguish pyridine from water and thiophene from mineral oil, although median detection and recognition thresholds obtained by nasal inhalation or gentle nebulization were significantly raised above normal. None recognized the characteristic odor of any vapor presented to him by nasal inhalation or gentle neublisation.

Within six months after operation subjective reports of olfactory discrimination were made by some patients. They commonly stated that they could recognize the vapors of gasoline and other distinctive vapors and of some distinctive foods--e.g., bacon frying. Objectively, detection and recognition thresholds for vapors obtained by inhalation into the oropharynx were significantly lower than those obtained by inhalation or nebulisation into the nasal cavity, but still significantly higher than thresholds for these same vapors obtained in normal subjects by nasal inhalation. These oropharyngeal thresholds generally required a longer time to obtain than did thresholds obtained by nasal inhalation. Objectively, most patients could detect and recognize the vapors of pure amyl acetate and camphor, although thresholds for each of these vapors were significantly elevated above normal.

Inhalation into the laryngeal stoma produced no olfactory response per se, although patients could detect differences between solvent and concentrated solutions of solutes such as pyridine and nitrobenzene.

Subjectively, each patient with a tracheostomy tube in place but without other surgical interference with any structure in the oropharynx or larynx noted no significant alteration in olfactory acuity. Detection and recognition thresholds for each of the vapors tested by nasal inhalation in these patients were within normal limits. Inflation of the rubber cuff surrounding the tracheostomy tube had no effect on subjective olfactory acuity, and olfactory thresholds were within normal limits. However, after laryngectomy subjective and objective olfactory acuity was significantly decreased in each patient.

Median detection and recognition thresholds for representatives of each of the four modalities of taste were also generally elevated in these patients. In addition, none recognized the flavor of pure banana and only a few recognized the flavor of pure orange.

Significance to Bio-medical Research and Program of the Institute:

The mechanism responsible for the hyposmia following laryngectomy is not clear. It is not related to surgical interference with the first cranial nerve, since surgery did not involve any anatomical disturbance of that area. It is unlikely that the hyposmia is related to a physiological alteration in the olfactory epithelium, since it was noted subjectively and measured

objectively as soon as two hours after operation. It is doubtful whether the olfactory epithelium would be significantly damaged within this time under the conditions of the surgical procedure. It is also doubtful whether the hyposmia is related to a significant alteration in air-flow, since gentle nebulisation of vapors into the area of the olfactory epithelium did not significantly lower the elevated detection or recognition thresholds determined by nasal inhalation. Furthermore, when normal air-flow was diverted by the placement of a tracheostomy tube, subjective reports of normal olfactory acuity and measurements of thresholds for vapors were within normal limits in each of the patients studied. In addition, subjective reports and objective threshold measurements for vapors were within normal limits after inflation of the tracheostomy cuff, producing an air-flow identical with that of a laryngectomized patient but without surgical interference to the larynx. Thus the hyposmia is apparently a complication of surgical interference with the larynx rather than the result of any significant alteration in air-flow to the olfactory receptors.

Certainly if vapors carried by air do not reach the olfactory receptors of the olfactory epithelium (the primary olfactory area) or those of the oropharynx and larynx (accessory olfactory areas) and make contact with these receptors, olfaction does not take place. However, effective air-flow is not substantially altered by either laryngectomy or tracheostomy. After laryngectomy air-flow can be maintained by air swallowing into the esophagus, while after tracheostomy it can be maintained both by normal air movement and by air swallowing. The exact volume of air moved by a laryngectomized patient past the pharynx by air swallowing into the gastrointestinal tract is not known. Judging from the intensity of muscular movements associated with this maneuver, this volume may be significant.

One hypothesis to explain the hyposmia after laryngectomy is that surgical interference with sensory nerves to the larynx at the time of its removal may significantly alter olfactory acuity through some complex feedback mechanism or by interfering with a diffuse anatomical projection system from pharynx and/or larynx to the olfactory brain. A phylogenetic relationship between pharyngeal and laryngeal function and olfaction has been identified among lower animals, and this interaction might play some role in man. The glossopharyngeal and/or vagus nerves may participate in some complex sensory laryngeal-olfactory feedback system; projections from the vagus nerve have been reported to terminate in the rhinencephalic groove and in the amygdala. These observations have clinical bearings. Since patients uniformly develop hyposmia after laryngectomy the surgeon who performs this procedure should inform the patient of this anticipated complication before he operates.

Failure to do so might further handicap the patient who may be aware only of his impending loss of speech, and who may not be psychologically prepared for the sudden loss of olfaction and the inability to obtain flavor from food.

Proposed Course of Project:

A continued cooperative effort is planned, studying all patients who have any

physical alteration of their head and neck anatomy, in order to better identify the true mechanisms of taste and smell.

Project Description:

Part II. Specific cooperative studies

E. Splenectomy and its role in chronic myelogenous leukemia

Objectives:

To assess the effect of splenectomy on tolerance to specific therapy and normalization of blood cell kinetics in patients with chronic myelogenous leukemia.

Methods Employed:

Patients with chronic myelogenous leukemia in remission, regardless of their prior treatment are included in this study. After appropriate base line studies, splenectomy and other indicated intra-abdominal procedures is performed. Several significant individual changes that have been observed in patients studied preliminary to this protocol serves as guidelines for assessing the effect of splenectomy in these patients. These include subjective symptomatic improvement, increased tolerance to subsequent specific hemotherapy and radiation therapy and improvement in blood cellular kinetics. The effect of splenectomy on these perameters is determined by appropriate statistical analyses which consider the effect of subsequent specific therapy.

Major Findings:

In the patients thus far studied, there have been significant improvement in symptoms, and enhanced tolerance of subsequent therapy. Study of additional patients will eventually allow the determination of the fact of splenectomy in favorably altering the clinical course and improving survival in patients with this disease.

Significance to Bio-medical Research and Program of the Institute:

The mounting clinical evidence for improvement in the clinical course of patients with chronic myelogenous leukemia and lymphoreticular disorders with splenectomy should lead to exploration of the mechanisms involved in this response. Such information will be of value to all involved in the exploration of improved therapeutic approaches to the treatment of this disease.

Proposed Course of Project:

The project will continue until the effect of splenectomy on all perameters mentioned is determined. On the basis of the findings so far, a reasonable

prediction is that the procedure will become a standard clinical adjunct in the treatment of chronic myelogenous leukemia.

TABLE 1
Individual Written Intramural Consultations Received by Surgery Branch, NCI
Listed by Institutes
1963-1970

	NCI	NINDS	NIAMD	NHLI	NIAID	NIMH	NIDR	EHS	TOTAL
1963	366	184	221	228	158	37	26	-	1220
1964	356	121	207	201	104	33	24	-	1046
1965	429	129	216	277	119	39	31	-	1240
1966	362	271	201	197	86	59	11	7	1205
1967	343	215	245	166	85	61	17	-	1132
1968	278	143	192	203	111	54	25	7	1013
1969	314	131	248	207	128	45	31	10	1114
1970	199	59	137	104	96	41	12	7	655

TABLE 2
Summary of Written Extramural Consultations Requested by or Through
Surgery Branch, NCI
1963 - 1970

	ENT	General Surgery	Gynecology	Urology	Oncology	Total
1963	346	14	7	18	9	394
1964	233	6	4	4	3	242
1965	268	6	2	9	1	286
1966	271	8	4	13	2	298
1967	294	11	1	1	1	308
1968	306	8	1	1	1	317
1969	329	8	2	3	2	344
1970	778*	12**	2	2	0	794

* 52% of these consultations were at the request of Dr. Robert I. Henkin, NHLI,ET, part of his clinical research program.
** Plastic surgery, 10
Thoracic surgery, 2

TABLE 3

Annual Report of NIH Surgical Suites
Listing the Number of Major & Minor Procedures
Carried Out by Each of the Operative Units at NIH
April 1, 1970 - March 31, 1971

Patient		NCI	NHLI	NINDS Neuro.	NINDS Muscle	NIAID	NIAMD	NEI	NIMH	NICHD	NIDR	Total # Pt. Operated		Cons.
NCI	Major	279	3	2	0	0	0	1	0	3	2	295	628	5
	Minor	295	1	2	0	0	0	0	0	8	2	333		25
NHLI	Major	30	257	0	0	0	0	0	0	0	1	288	390	0
	Minor	52	40	0	4	0	0	0	0	2	0	102		4
NINDS	Major	7	2	104	0	0	0	4	0	0	1	119	346	1
	Minor	10	0	14	198	0	1	1	0	1	2	227		0
NIAMD	Major	46	6	3	0	0	0	1	0	2	0	63	103	5
	Minor	24	5	0	5	0	1	0	0	0	0	40		5
NIAID	Major	16	3	3	0	0	0	0	0	0	0	25	61	3
	Minor	23	5	2	4	0	0	0	0	0	1	36		1
NIDR	Major	0	0	0	0	0	0	0	0	0	3	3	5	0
	Minor	2	0	0	0	0	0	0	0	0	0	2		0
NIMH	Major	2	1	0	0	0	0	0	0	0	0	4	5	1
	Minor	1	0	0	0	0	0	0	0	0	0	1		0
NICHD	Major	2	0	0	0	0	0	0	0	4	0	6	24	0
	Minor	9	0	0	0	0	0	1	0	8	0	18		0
NEI	Major	4	0	0	0	0	0	30	0	0	0	34	76	0
	Minor	2	0	0	4	0	0	36	0	0	0	42		0
GRAND TOTAL		804	323	130	215	0	2	74	0	28	12	1638	1638	50

2401

TABLE 4

Number of Surgical Procedures Performed
By NCI Surgery Branch Staff
and Magnitude of the Procedures in 1970

	10 East	Other NCI	Other Institutes	Total
1963	470	159	399	1028
1964	424	215	256	895
1965	394	112	199	705
1966	410	113	218	741
1967	440	145	140	725
1968	394	205	113	712
1969	339	233	158	730
1970	301	273	230	804
Major	202	101	83	386
Minor	99	172	147	418

Publications:

Wolfe, S.M., Harrah, J.D., Merritt, W.R., Hoye, R.C., and Gordon, R.S.: An effective method for radiographic diagnosis of Mechel's diverticulum. J.A.M.A. 205: 801-802, 1968.

Jowsey, J., Hoye, R.C., Pak, C.Y.C., and Bartter, F.C.: The treatment of osteoporosis with calcium infusions: Evaluation of bone biopsies. Amer. J. Med. 47: 17-21, 1969.

Johnson, R.E., Thomas, L.B., and Chretien, P.B.: Correlation between clinico-histologic staging and extranodal relapse in Hodgkin's disease. Cancer, 25: 1071-1075, 1970.

Hoye, R.C., Ketcham, A.S., and Henkin, R.I.: Hyposmia following paranasal sinus exenteration or laryngectomy. Amer. J. Surg., 120: 485-491, 1970.

Deckers, P.J., Fraley, E.E., Paulson, D.F., and Harbert, J.C.: Vascular obstruction of the superior renal infundibulum in children. Surgery, 67: 856-862, 1970.

Neel, H.B., III, Neel, H.B., and Hammond, W.G.: A reappraisal of primary gallbladder carcinoma. Journal of Surgical Oncology, 2: 131-143, 1970.

Doppman, J.L., Hammond, W.G., Aurbach, C.D., and Pak, C.Y.C.: The anatomic basis of parathyroid venous sampling. Radiology 95: 603-610, 1970.

Banfield, W.G., Grimley, P.M., Hammond, W.G., Taylor, C.M., deFlorio, B., and Tousimis, A.J.: Electron probe analysis for iodine in human thyroid and parathyroid glands, normal and neoplastic. JNCI 46: 269-273, 1971.

Articles in Press:

Morton, D.L., Glancy, D.L., Joseph, W.L., Adkins, P.C., and Johnson, R.E.: Management of radiation-induced pericarditis with effusion. Ann. Thor. Surg. in press.

Catalona, W.J., Engelman, K., Hammond, W.G., and Ketcham, A.S.: Familial medullary thyroid carcinoma, Pheochromocytoma and parathyroid adenoma (Sipple's Syndrome): Study of a kindred. Cancer, in press.

Henkin, R.I., Schechter, P.J., Hoye, R.C., and Mattern, F.T.: Idiopathic hypogensia with dysgeusia, hyposmia and dysosmia: A new syndrome. J.A.M.A. in press.

Serial No. NCI-3801

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of Cancer Surgery as to its Effectiveness and Safety in Providing Definitive and Therapeutic Care of Cancer Patients

Previous Serial Number: Same

Principal Investigators: Alfred S. Ketcham, M.D. and Entire Surgery Branch, NCI

Other Investigators: L.B. Thomas, M.D., S. Kakehashi, M.D. K.I. Cohen, M.D., R. Johnson, M.D.

Cooperating Units: Laboratory of Pathology, NCI, Dental Service Branch, Experimental Therapeutics Branch, NHLI; Radiation Branch, NCI, Diagnostic Radiology Department, Pathologic Anatomy Department, Clinical Pathology Department, Nursing Department, Anesthesiology Department, Social Work Department, Rehabilitation Department, Clinical Center; NINDB; NIDR.

Man Years: (July 1, 1970 through June 30, 1971)

Total:	8-1/2
Professional:	5
Other:	3-1/2

Project Description:

Part I. Study of the Biologic Behavior and Results of Surgical Therapy of Cancer of the Uterine Cervix.

A. Pelvic Exenteration for Carcinoma of the Cervix

B. Scalene Nodes as Indicators of Disseminated Cancer

Part II. Study of the Biologic Behavior and Results of Surgical Therapy of Cancer of the Head and Neck Regions

A. A Retrospective Computer Analysis of Patients with Head and Neck Tumors Treated Surgically at the NIH

B. Sarcomas of the Head and Neck

Part III. The Role of Bacterial Infection in Cancer Surgery

Part IV. The Evaluation of Uretero-ileostomy in Urinary Diversion

Part V. Axillary Dissection with Preservation of the Pectoralis Major Muscle

Part VI. Extended Deltopectoral Flap Reconstruction of Defects of the Head and Neck Region

Part VII. The Development and Evaluation of the Technique of Reconstruction of Defects of the Head and Neck Utilizing an Extended Shoulder Flap

Part VIII. Hemipelvectomy: Its Role in the Treatment of Extremity Sarcoma

Part IX. The Surgical Treatment of Melanoma

Part X. Vascular Complications of Radical Cancer Surgery of the Pelvis and Groin

Part XI. Evaluation of Pulmonary Resection for Metastatic Disease in Patients with Apparent Control of Primary Lesion

Part XII. Significance of Tumor Doubling Time in Evaluating Operability in Pulmonary Metastatic Disease

Part XIII. The Use of Coumadin Anticoagulation as an Adjunct to the Surgical Treatment of Sarcomas

Project Description:

Part I. Study of the biologic behavior and results of surgical therapy of cancer of the uterine cervix

A. Pelvic Exenteration for Carcinoma of the Cervix

Objectives:

To evaluate a 15 year experience with pelvic exenteration for carcinoma of the cervix and define the value as well as the limits of the procedure.

Methods Employed:

Over 2,100 patients with carcinoma of the cervix have been seen as outpatients and evaluated in the screening clinic to determine the feasibility of treatment. Approximately 535 were admitted for study and came to surgery with the preoperative impression that their disease was confined to the pelvis.

Pelvic exenteration was required to remove the cancer, in 165 patients, 68 of whom had never previously been treated and were preoperatively considered to be advanced, Stage III or IV, and 97 of whom were previous radiation failure patients.

Major Findings:

The major points of interest including preoperative finding, complications, morbidity, mortality, and survival, are detailed in the accompanying tables.

Significance to Bio-medical Research and Program of the Institute:

This is the highest percent salvage of any report found in the literature for advanced cervical carcinoma, either primary or radiation recurrent. The high mortality of 17% (28 patients) from 1 to 108 days postoperative, is indicative of not only the magnitude of the procedure but the relatively unrestrictive preoperative limitations that we have placed upon those patients who are offered this type of aggressive surgery. The intra-operative 30-day mortality is 7%. Selected individuals are offered vaginal reconstruction as part of their overall rehabilitation. Continued improvements and innovations in the care of the urinary ileostomy and colostomy stomas are being developed. A better understanding of the surgical physiological principles of stress is an extremely important ancillary program resulting from this study.

Proposed Course of Project:

While continuing to review the indications for surgery and to investigate the reasons for failure even by radical surgery, we are trying to determine more specific and reliable criteria for operability and curability without extending the scope of surgical dissection. It has definitely been shown that unilateral leg edema and sciatic distribution of leg pain are reliable signs of incurability. Lymphangiography has become a research tool used to direct the surgeon's attention to possible areas of lymph node metastasis. In addition, the inadvisability of posterior pelvic exenteration as treatment for cervical cancer has been shown.

TABLE I

CARCINOMA OF THE CERVIX TREATED BY PELVIC EXENTERATION
FIVE-YEAR CUMULATIVE PERCENT SURVIVAL

OPERATION PERFORMED	PRIMARY CARCINOMA PELVIC LYMPH NODES			RECURRENT CARCINOMA PELVIC LYMPH NODES			ALL CASES TREATED PELVIC LYMPH NODES		
	POSITIVE	NEGATIVE	TOTAL	POSITIVE	NEGATIVE	TOTAL	POSITIVE	NEGATIVE	TOTAL
ANTERIOR PELVIC EXENTERATION (A.P.E.)	56%(9)*	47%(18)	51%(27)	50%(2)	43%(7)	44%(9)	55%(11)	47%(25)	50%(36)
TOTAL PELVIC EXENTERATION (T.P.E.)	50%(17)	44%(24)	46%(41)	7%(19)	31%(62)	25%(81)	27%(36)	36%(89)	34%(125)
POSTERIOR PELVIC EXENTERATION (P.P.E.)	-	-	-	-	-	-	-	100%(1)	100%(1)
TOTAL - ALL EXENTERATIONS (A.P.E.+T.P.E.+P.P.E.)	52%(26)	46%(42)	48%(68)	11%(21)	33%(69)	28%(90)	34%(47)	39%(115)+	38%(162)

* number within parentheses indicates number of cases in each group.
\+ includes four cases with a negative preoperative biopsy and no tumor in specimen.

2407

Serial No. NCI-3801

TABLE 3

PRIMARY CAUSE OF OPERATIVE DEATH IN 28 OF 162 PATIENTS TREATED BY PELVIC EXENTERATION FOR CARCINOMA OF THE CERVIX

CAUSE OF DEATH	PRIMARY CARCINOMA	RECURRENT CARCINOMA	TOTAL DEATHS	DAY OF DEATH FOLLOWING SURGERY
SEPSIS	5	12	17	
SEPTICEMIA	1	4	5	6,35,43,62,93
PERITONITIS	1	3	4	31,43,53,57
FISTULAE	1	2	3	14,87,108
RENAL FAILURE	-	2	2	57,62
PNEUMONIA	2	1	3	14,44,90
CARDIAC FAILURE	-	2	2	7,7
PULMONARY EMBOLISM	-	4	4	4,7,8,28
ACUTE HEMORRHAGE	1	3	4	1,1,5,62
UNKNOWN	1	-	1	66
TOTAL DEATHS/TOTAL TREATED	7/68(10%)	21/94(22%)	28/162(17,3%)	

Deaths less than 30 days = 12/162 = 7.4%

TABLE 2

CORRELATION OF THE MEIGS' SURGICAL PATHOLOGIC STAGING WITH SURVIVAL
OF 162 PATIENTS WITH CARCINOMA OF THE CERVIX WHO UNDERWENT PELVIC EXENTERATION

	5-YEAR CUMULATIVE PERCENT SURVIVAL		
PATHOLOGIC STAGING	PRIMARY CASES	RECURRENT CASES	ALL CASES
$A + A_o$	75% (5)*	43% (7)	60% (16)
B	56% (10)	0% (14)	33% (24)
C	31% (11)	49% (25)	44% (36)
D	60% (10)	17% (6)	44% (16)
E	43% (32)	18% (38)	30% (70)

* number in parentheses indicates total cases in each group.

2409

TABLE 4

CORRELATION BETWEEN HOSPITAL MORTALITY, OPERATIVE TIME AND TOTAL INTRAOPERATIVE BLOOD REPLACEMENT

OPERATIVE HOURS	NO. OF PATIENTS	PATIENTS DYING IN THE HOSPITAL	INCIDENCE OF DEATH	AVERAGE BLOOD GIVEN INTRAOPERATIVE
4-6 hrs	47	7	15%	2571 cc
6-8 hrs	67	6	9%	5250 cc
8-10 hrs	34	10	26%	7650 cc
10-12 hrs	11	3	27%	9750 cc
12-15 hrs	3	2	66%	>10,000 cc

B. Scalene nodes as indicators of disseminated cancer

Objectives:

To determine the instance of tumor dissemination, from primary pelvic cancer, to the scalene node bearing areas.

Methods Employed:

All patients with biopsy proven cancer of the structures of the female pelvis who are candidates for definitive curative surgery will have left scalene node biopsy. The procedure is carried out only in the absence of distal tumor spread. A generous pad of lymph node bearing tissue from the left subclavicular fossa is obtained from microscopic analysis. Using local anesthesia, this minor operative procedure can be carried out without complications.

Major Findings:

Sixty nine patients with carcinoma of the cervix whose preoperative evaluation revealed no evidence of cancer spread beyond the confines of the pelvis and who were candidates for surgery, had scalene lymph pad tissue removal. Eight of these patients were found to have unsuspected metastases to one of the lymph nodes in the subclavicular fossa. None of these patients had any evidence of pulmonary tumor or other metastases. They were, therefore, deemed incurable by surgery and a radical procedure avoided which would only have been palliative.

Significance to Bio-medical Research and Program of the Institute:

A significant number of patients developed metastatic disease at such a time interval following primary tumor treatment that there is the suggestion that the metastases was present preoperatively. No matter how complete and extensive the pretreatment evaluation, the surgeon must face this distressing situation only too often. The identification of metastatic disease from pelvic carcinoma to unsuspected anatomical areas adds another preoperative modality of determining what patient may be free of cancer metastases at the time of definitive surgery.

Proposed Course of Project:

Continue study of all patients admitted for treatment of primary pelvic disease to rule out previously unrecognized metastases.

Project Description:

Part II. Study of the biological behavior and results of surgical therapy of cancer of the head and neck regions.

Objectives:

1. To determine the feasibility of an aggressive surgical approach to irradiation or surgical recurrent malignancies of the head and neck areas.

2. An evaluation of adjunctive immediate preoperative irradiation in previously untreated cancer of this anatomical area.

3. An evaluation of adjunctive chemotherapy used before surgery and its long-term intermittent use after surgery.

4. To determine the effectiveness of a combined intracranial facial approach to cancer of the paranasal sinuses.

5. To determine the prevalence, site of origin, and rationale of treatment of unknown primary disease, metastatic to head and neck areas.

6. Evaluation of new methods of surgical approach and reconstruction in surgical treatment of head and neck cancer.

7. The clinical pathological correlation of epidermoid carcinoma of the head and neck area.

Methods Employed:

Following extensive preoperative clinical, pathological, and roentgenological studies standard and new radical surgical procedures are carried out.

(a) In selected patients with carcinoma of the pharynx or larynx, especially in those with positive nodes in the neck combined pre- and postoperative irradiation will be carried out. At the time of surgery either unilateral or bilateral neck dissection will be performed in continuity with resection of the main tumor mass. Concomitant control series will also be included to evaluate the effects of the radiotherapy in increasing survival and decreasing the incidence of local recurrence of disease.

(b) In patients with Stage II, III, and IV epidermoid cancers of the oral cavity treatment with methotrexate will be used for two to three weeks before surgery and after surgery will be used on a long-term intermittent basis. Surgery will involve wide resection of the primary tumor with unilateral or bilateral neck dissection. An effort will be made to increase survival and decrease the incidence of local recurrence with this combined chemotherapy and surgery approach.

(c) In patients having paranasal sinus cancer, either primary or recurrent which suggests roentgenographically or clinically that the cribriform plate will be removed at the time of surgery, our approach from an intracranial and combined facial surgical incision which will permit en bloc removal of the tumor and at the same time affords protection of the surrounding vital structures.

(d) In patients requiring surgery to control recurrent disease following previous therapy treatment will be combined pre- and postoperatively with a chemotherapy approach. During our continuing studies for inpatients with head and neck cancer some 70-80% of the patients screened for admission have been failures with previous therapy, usually radiation. The actual incidence of patients admitted and treated who have been previous failures is 71%. By using chemotherapy before and after surgery it is anticipated that an improvement in survival results will be realized.

(e) In patients requiring large resection for treatment of their cancer immediate plastic reconstruction will be carried out using flaps of tissue from the pectoral or shoulder areas. These flaps will be used singly or in combination at the time of the resection.

Major Findings:

The appearance of recurrent local disease, if treatment has not been successful, is noted within 18 months in over 75% of the incidences and within 6 months in 35% of the incidences. There has been a significant correlation between local recurrence and failure of further treatment. The patient who develops rapidly recurrent malignant growth in the operative field, can be offered little survival by further treatment. There has been no correlation between local recurrence and distance spread and a 51% incidence of the distance metastasis following what is thought to be definitive surgery. Seventy-nine patients have been included in the preoperative irradiation study. The concept of a small dose of irradiation preventing tumor dissemination of actively viable cells at surgery is based upon animal studies carried on in this Institute and has been underway clinically for the past eight years. Review of the series indicates that there may be little effect obtained by this dose of preoperative irradiation in decreasing the incidence of wound recurrence following surgery and the study has been halted while an evaluation is made of the results. Morbidity related to the radiation has been significantly increased over patients who received surgery without preoperative radiation.

The combined intracranial facial operation for cancer of the paranasal sinuses has been performed in 48 patients to date. A review of the results revealed that of the patients treated, 19 were dead and 29 were living for a time interval of four months to 11 years followup. The operation has been extended to include patients showing some involvement of the cerebral meninges. All of the patients who have thus far been approached in this manner, have had extensive disease and have not been

amenable to treatment in any other fashion. Any survival at all is indeed promising, but more patients and more time is necessary before the procedure can be recommended for routine use. The advantages of the combined procedure are that it involves (1) evaluation of intracranial disease, (2) protection of the brain, (3) avoidance of spinal fluid fistulas, (4) more adequate hemostasis, (5) facilitation of resection, and (6) en bloc resection.

Unknown primary lesions with neck metastases is not a frequent occurrence and accumulation of a series of significant numbers is difficult. The eventual discovery of the primary lesion has usually been in the nasopharyngeal sinus area, but three cases have been from the thyroid and one from the kidney. In the past year, six new cases have been admitted to this study.

Significance to Bio-medical Research and Program of the Institute:

These locally resistant, usually recurrent head and neck lesions have long been a problem to oncologists. We are attempting to correlate aggressive surgical exploration of these lesions with survival and the mode of tumor dissemination. Careful environmental background reviews reflect the close relationship between the Surgery Branch and the Dental Prosthetic Unit allowing a comprehensive evaluation of the indications for plastic reconstruction of facial deformities and evaluation of newer prosthetic materials.

Proposed Course of Project:

The extent of surgical excision cannot be much increased in its scope beyond that which we are now able to perform successfully. More patients are necessary before the combined cranial-facial approach to paranasal sinus cancer can be recommended for routine use. Both procedures are based on sound experimental and clinical observations and final evaluation should prove rewarding. Pilot studies are under way to determine the feasibility of intensive preoperative medication in patients with disease which has locally extended beyond that which can be cured by conventional means.

A. A retrospective computer analysis of patients with head and neck tumors treated surgically at the NIH

Objectives:

One of the least refined areas of cancer biology concerns the ability of the physician to assign prognostic significance to laboratory and clinical data. Presently clinical and histologic staging are the only reliable criteria now utilized to predict patient survival and even with these criteria there is considerable variability. With the multiple diagnostic and

and evaluative tests now available, an effort is being made in this study to determine other predictive factors relative to survival in the head and neck cancer patients. Available evidence indicates the defence mechanism which might be immunologically mediated in the host, might be of prime importance in achieving long term survival from many different malignancies. There has been considerable recent interest in the prognostic significance of lymph node reactivity of patients with carcinoma of the breast. Correlative value has been ascribed to bilaterally palpable axillary nodes and the cellular structures of nodes examined. A similar study is under way with patients treated surgically for cancer of the larynx.

Since cancer of the head and neck represents a variety of diseases, the patients have been assigned to traditional categories based on location of the primary tumor for the purpose of comparing our survival data with other series. The first group to be studied were those patients with cancer of the larynx and hypopharynx. Patients with tumors of the oral cavity, nasal cavity, paranasal sinuses, salivary glands, skin and unknown primary sites will be studied subsequently.

Methods Employed:

A detailed retrospective study of all head and neck cancer patients treated surgically at NIH will be undertaken. To date 84 patients with cancer of the larynx have been studied. Approximately 200 clinical and histological variables extracted from the records of each patient was submitted for computer analysis using the data processing program of the cancer end results section.

Major Findings:

The majority of patients studied had either very extensive primary disease or radiation recurrent cancer. Of the 84 patients, 44 had intrinsic primary laryngeal tumors, 20 had extrinsic primary disease and 20 patients had radiation recurrent or persistant tumors unresponsive to therapy. The involvement of cervical nodes was taken as the prognostic standard against which all other variables were compared. Intrinsic lesions with negative nodes had a 71% 5-year survival while positive nodes indicated a cumulative 5-year survival of 33%. As expected, positive nodes were more likely to occur with extrinsic lesions (63% vs. 24% for intrinsic lesions) and resulted in a drop in 5-year survival from 49% for patients with negative nodes to 30% with positive nodes. Other less recognized criteria were equally significant. Of the 13 patients with cytologically positive intraoperative wound washings only two were alive at five years. The likelihood of cure was considerably greater in those patients who had either a unilateral or bilateral elective neck dissection (19 patients 78% survival) than in those patients who had a neck dissection for clinically positive nodes (30%) or in those who had clinically negative nodes and no primary

neck dissection (46%). If there was a positive intra-operative frozen section margin for which additional tissue had to be taken to assure a clear margin, the total cumulative 5-year survival dropped from 54% for those with initially clear margins to 13% for those with at least one positive frozen section margin. Of the 11 patients with microscopically positive margins on permanent section the 5-year survival was 28%. Multiple preoperative variables were likewise statistically evaluated and found to be significant in predicting ultimate survival. For example, in patients with preoperative serum albumin levels of less than 3 mgm %, the cumulative 5-year survival was 29% while those with albumin levels of 3.9 mgm % or greater had a 75% likelihood of living 5 years.

Significance to Bio-medical Research and Program of the Institute:

It is apparent that there are numerous clinical tests and other less utilized variables which have prognostic significance in predicting long term survival in patients treated surgically for cancer of the larynx. Hopefully, similar variables will be identified in other head and neck cancers as this project proceeds.

Proposed Course of Project:

The remainder of the patients with head and neck cancer treated at the NIH will be studied retrospectively using the same computer program for data processing.

B. Sarcomas of the head and neck.

Objectives:

To evaluate the experience of treating the rare, but highly lethal, sarcom of the head and neck area.

Methods Employed:

Nineteen patients with sarcomas of the head and neck area have been treated by the Surgery Branch and followed for a minimum of four years. Each of these patients, except for one child with an embryonal rhabdomyosarcoma, had large tumors which had extensively destroyed adjacent normal structures. Ten were recurrent or persistent following previous surgery or radiation. Ten were treated by combined cranial-facial resection and four of whom the orbital contents were conserved.

Major Findings:

Seven patients died of their disease from three to eight months post-operatively, nine are living free of tumor, and three have died free of cancer, but of complicating cardiovascular disease. There were no operative deaths.

Significance to Bio-medical Research and Program of the Institute:

Through an aggressive surgical approach to sarcomas of the head and neck, a four to ten year cure rate of 50% may be provided even to patients with lesions arising or extending into the ethmoid sinuses. Cribriform plate resection for these large, usually recurrent lesions, is carried out through a combined cranial-facial approach. Although massive tissue resection is often mandatory, with the truly en bloc type of resection provided by this approach, acceptable cosmetic results are obtained by preoperative reconstructive planning.

Unawareness of the propensity of these tumors to recur after marginal excision and an unwillingness to create a cosmetic deformity or attempts to cure by radiotherapy alone, or even by chemotherapy, accounts in great part for the low cure rates often reported. The frequent histological classification of unclassified, undifferentiated and undetermined types of biopsied sarcoma and the frequency with which diagnoses are changed, suggests that the inaccuracy of specific diagnosis should not be a critical factor in determining therapy. Radiation and chemotherapy have not generally been shown to offer advantages over surgery for sarcoma of the head and neck region.

Proposed Course of Project:

Continued evaluation of all modalities of treatment for sarcomas of head and neck will be carried out. Attempts will be made to indoctrinate the medical community about the increased salvage that may be obtained in these patients by first, early recognition and secondly, aggressive therapy.

Project Description:

Part III. The role of bacterial infection in cancer surgery.

Objectives:

To further substantiate findings in the previous years of the evaluation of the operative wound infection rate and its relation to the "prophylactic" use of antibiotics following extensive cancer surgery.

Methods Employed:

By immediate admission and repeated preoperative culturing of all patient's nose, skin and tumor, patients are classified into either carrier or non-carrier status of hemolytic staphylococcal aureus coagulase positive organisms. By bacteriophage typing, through the cooperation of the Clinical Pathology Department, a correlation could be drawn between the patient's preoperatively carried organism and that carried postoperatively,

either asymptomatically or related to a postoperative infection. In previously reported studies patients undergoing radical cancer surgery had, on a double blind basis, either ten days of Chloromycetin postoperatively, or a placebo. A subsequent study entailed the use of seven days of Chloromycetin postoperatively in all patients, while on a double-blind basis, the patients received preoperatively for ten days, either three days of placebo or three days of antibiotic. The current protocol utilized intravenous antibiotics before, during and seven days following the operation. Following two separate studies, which have been reported, antibiotic therapy has been used prophylactically in a therapeutic manner on all patients undergoing cancer therapy. These patients have been carefully observed for wound infection and any possible correlation to their "carrier status" or pathogenic organisms.

Major Findings:

The previous annual reports detail the specific results of both double-blind antibiotic studies. In brief, the infectious complication rate following extensive cancer surgery was lowered from 54% to 14% by the use of pre- and postoperative antibiotics rather than placebo. An 8% infection rate occurred when the intraoperative use of antibiotics was employed in addition to pre- and postoperative coverage.

The overall results for the last 14 years can be seen in the accompanying table. The 1960 rate was elevated to 32% in that this was the year of the original study, whereas 1959 was lowered somewhat because of the discontinuance of the use of penicillin and streptomycin and the selection of antibiotics was left to the discretion of the surgeon.

14 YEAR SUMMARY OF INFECTION RATES AND
STAPHYLOCOCCAL INCIDENCE FOLLOWING EXTENSIVE CANCER SURGERY

Year	Extensive cancer operations	Infection rate per cent	Per cent of total infections that were staphylococcal
1956....	128	28	35
1957....	139	31	42
1958....	145	30	59
1959....	130	22	69
1960....	144	32	74
1961....	115	13	73
1962....	132	10	72
1963....	137	15	70
1964....	131	7	51
1965....	132	6	36
1966....	141	5	22
1967....	128	6	17
1968....	209	8	14
1969....	217	10	8
1970....	132	11	7

During the past 15 years, 2,408 extensive cancer operations have been performed on the Surgery Branch of the National Cancer Institute. During the early part of this study the infectious complications ranged from 22% to 31% during a time when penicillin and streptomycin were used somewhat routinely at the discretion of the surgeon. The results of a double-blind antibiotic-placebo study revealed an infection rate of 54% when a placebo was used and 14% when routine ten-day postoperative antibiotic therapy was instituted. A subsequent double-blind study showed that by giving drug for three days preoperatively and seven days postoperatively, antibiotic therapy resulted in a 17% rate of infectious complications. With continued use of antibiotic prophylaxis, while continuing the same type of extended operative procedures, the infection rate was 10% and 15%. Chloramphenicol, 2 gm. every six hours, was the antibiotic used and there were no significant complications referable to drug toxicity and only five severe infectious complications referable to lose of staphylococcal sensitivity to the drug.

Over the past years the percentage of total infections that were hemolytic staphylococcal aureus DNA-ase positive in origin progressed from 35% in 1956 to 70-75% in 1961-1964, and dropped to 36% in 1965 and 14% in 1968. At the same time the incidence of patients who were carriers of staphylococci on admission to the hospital was 30% and has remained at the approximate level. Patients who carry staphylococci into the operating room, usually asymptomatically, run at 38% increased rate of infection over non-carriers. Ninety-three percent of these patients will become infected with the same phage typed organism that they carried into the operating room. This suggests that self-infection may play as an important a role in infectious complications as does cross contamination. Of greater importance at this time is the alarming increase in the complication associated with Pseudomonas and Klebsiella and E.Coli infections. So frequently, death is the result of a septicemia from these organisms. They are much more or a problem today, both in prevalence and in morbidity and mortality than the staphylococcus.

During the past four years, 12 to 24 hours of preoperative antibiotics have been used routinely along with continuous intraoperative antibiotic drugs and three to seven days of postoperative therapy. The overall infectious experiences of patients undergoing very radical cancer surgery is seen in the next table.

POSTOPERATIVE INFECTIONS COMPLICATION RATE
FOLLOWING DIFFERENT MODES OF ANTIBIOTIC THERAPY

	# of Pts.	Infections	Rate
Antibiotic used at discretion of surgeon	704	197	28%
No antibiotic therapy	87	46	53%
Postoperative therapy	332	66	20%
Pre- & postoperative therapy	288	40	14%
Pre-, post, and intraoperative therapy	802	88	11%

Significance to Bio-medical Research and Program of the Institute:

When postoperative morbidity and mortality are increased due to complications related not directly to the cancer but due to infection, the overall evaluation of surgery and its place in cancer therapy is markedly altered. Bringing an alarming infection rate under relative control has decreased morbidity and mortality as indicated in the previous paragraphs. In a hospital such as this where rather elaborate facilities are available for close patient surveillance, studies such as this can be done which can be of significant value to the practicing surgeon. The fact that the total infectious complication rate seems to be rising, may be related to the reluctance on the part of the clinician to use Chloramphenicol which has been shown over the years to be the drug to which the hospital organisms are most susceptible. Because of the medico legal aspects related to Chloramphenicol administration, the resident staff use this antibiotic only on direct insistance on the part of the senior staff. The continued decrease in the staphylococcal infection complication rate is consistent with findings in most hospitals through the country.

Proposed Course of Project:

The indications for "prophylactic" antibiotic usage when carrying out the present clinical investigative studies within the Surgery Branch are unequivocable. Close surveillance of carrier patients and personal carriers within the hospital is being continued. It is anticipated that continuation of the surveillance program will not only lessen the so-called epidemic infectious outbreaks occasionally seen on all surgical wards but will allow more rapid and adequate control when such does occur. It is proposed that the critical period of antibiotic coverage is at the time of the operative procedure itself and that constant infusion and drug level maintenance of antibiotics during this period is mandatory in cancer surgery.

Part IV. Evaluation of uretero-ileostomy in urinary diversion

Objectives:

A continuing study of the physiology and bacteriology of the ileal conduit is being carried out in the Surgery Branch. Evaluation of function and infection of ileal urinary conduits has been carried out on patients whose urinary stream has been diverted during radical surgery for pelvic malignancy. Patients with non-visualization of one kidney due to ureteral obstruction by pelvic malignancy are being evaluated before and after ileal diversion by excretory urography and isotope renography. A recent interest in the use of the isolated segment of sigmoid colon is now being incorporated into this clinical study.

Major Findings:

We have found that persistent and significant upper urinary tract infection occurs more frequently in patients whose conduits show weak peristalsis and who carry residual urine greater than 15 ml. Obstruction of the conduit by constructions and sharp angulation also prolong the emptying rate and increase the incidence of urinary infection.

Kidneys which do not function because of ureteral obstruction usually regain some function on excretory urography after urinary diversion. However, the radioisotope renogram has shown that tubular function often remains very depressed, despite the appearance of adequate function on the IVP. We continue to divert such obstructed kidneys to preserve as much renal function as possible, regardless of duration of obstruction. However, this study has emphasized the importance of following carefully the contralateral normal kidney which carries most of the burden of total renal function in those patients whose previously obstructed kidney fails to regain adequate tubular function after obstruction is relieved.

Significance to Bio-medical Research and Program of the Institute:

All aspects of urinary diversion after radical pelvic surgery continued to concern us, because the most common non-malignant cause of death after recovery from pelvic exenteration is due to renal obstruction or infection. It is hoped that sigmoid diversion may play an increased role in diversion in that it obviates one very critical intestinal anastomosis.

Proposed Course of Project:

Continued clinical evaluation of all techniques of diversion.

Project Description:

Part V. Axillary dissection with preservation of pectoralis major muscle.

Objectives:

Because of the severe disability resulting from the currently used techniques of axillary dissection, a reassessment of the operation was undertaken heretofore, two techniques of gaining access to the axilla were used, either excision of the muscle, which greatly weakens the upper extremity; or transection and resuture of the muscle, which partially devascularizes and denervates the muscle, requires prolonged immobilization after the operation and results in residual limitation of motion and weakness of the extremity.

Methods Employed:

To devise a technique of access to the axilla without transection of the pectoralis major muscle, experiments were conducted using normal volunteers, cadavers, and finally in patients having axillary dissections. It was found that a semi-lateral position of the patient with the arm adducted across the chest proved to give adequate exposure of the axilla contents without section of the muscle and without compromise of the goals of the operation. This unique position of the patient in turn required the development of a new technique of executing the operation.

Major Findings:

Thus far, axillary dissection utilizing this position and technique has been completed in twenty-six patients. With exception of two patients in which partial section of the innervation of the muscle was necessary because of bulky metastatic tumor deposits, the neurovascular bundle to the pectoralis major muscle was preserved in all patients. The average post-operative followup of the patients is fourteen months at present. In spite of the fact that fifteen patients had axillary lymph nodes involved with metastatic tumor and in eight of these the tumor had extended through the node capsule into the axillary areolar tissue, no recurrences have developed. All patients have had perineum healing of their wounds and have regained full motion of the extremity. In addition, edema of the upper extremity commonly seen after the conventional technique of axillary dissection utilizing excision of the pectoralis major muscle has not occurred in our patients.

Significance to Bio-medical Research and Program of the Institute:

Resection of the axillary lymph nodes is an established aspect of the treatment of tumors of the extremity and adjacent chest. The technique

of the operation described has proved to give a superior cosmetic and functional result in our patients, and has been of particular benefit in those patients who have required bilateral axillary dissections, most of whom were male laborers.

Proposed Course of Project:

A detailed illustrated description of the operation has been prepared, along with the analysis of the patients who have had the operation. This material will be submitted for publication in a surgical periodical, with the recommendation that the operation be adopted as a standard technique of axillary lymph node dissection.

Project Description:

Part VI. Extended shoulder flap reconstruction of defects of the head and neck region.

Objectives:

To develop a direct, easily accomplished, and reliable means of reconstruction of defects of the chin, anterior floor of mouth, and tongue, that offers a superior cosmetic and functional result than achieved with conventional techniques of reconstruction.

Methods Employed:

To date, no satisfactory means of reconstructing defects in the chin, anterior floor of mouth, and tongue have been devised. The cosmetic deformity resulting from resection of massive defects in this region and the functional disability, e.g., continuous drool, impairment of speech, prevention of mastication, and pulmonary aspiration of saliva led to an assessment of this heretofore unsolved problem. A flap having its base in the sternal region, and extending to a tip in the posterior axillary fold was developed for use in the reconstruction of these defects. The flap has been perfected so it can be raised and rotated without loss of viability of the tip and with minimum inconvenience to the patient.

Major Findings:

The extended deltopectoral flap as utilized in patients thus far, including two who had virtual complete loss of the free tongue, has allowed complete restoration of speech and swallowing in each case. The technique represents the first cosmetically and functionally satisfactory means of restoration of tissue in such defects.

Significance to Biomedical Research and Program of the Institute:

Impairment of function and the distortion of the appearance of patients having massive resections of tumors involving the chin and anterior tongue region has constantly presented a challenge to the members of the Surgery Branch as well as head and neck surgeons elsewhere. The use of this technique of reconstruction will markedly shorten the hospitalization time of these patients following removal of such malignancies, and allow a rapid and more complete return to their community with elimination of the nursing care required heretofore after such operations.

Proposed Course of Project:

Improvements in the technique of reconstruction utilizing this flap will be made so as to allow its use both at the time of resection of the primary tumor and in those patients who had had resection of the tumor at some time in the past and have residual, functional and cosmetic deficits. In addition, this flap has been used in combination with the extended shoulder flap in several patients who had full thickness losses of the face that would have been virtually impossible to construct with currently available techniques. Further improvements in the technique of reconstruction with such flaps should relieve the reticence of the surgeon to resect massive malignancies of the head and neck region which, though technically feasible, would create distortions of appearance and function that would deter the patient from accepting the operation.

Project Description:

Part VII. The development and evaluation of the technique of reconstruction of defects of the head and neck utilizing an extended shoulder flap.

Objectives:

With the premise that a major deterrent to the performance of radical operations in the head and neck region is the numerous prolonged and tedious operations requiring multiple procedures, each one entailing risk of loss of the tissue used in reconstruction and lengthy hospitalizations at great expense to the patient and the medical institution, a direct, reliable, and relatively easily executed technique of reconstruction of head and neck defects with an extended shoulder flap was attempted.

Methods Employed:

After a review of the conventional methods of reconstructing head and neck defects, it appeared that a flap having its base in the occipital region would provide tissue for reconstruction of the head and neck region without the necessity of secondary migrations of the pedicle, and, hence, entailing risk of loss of the tissue, especially patients who have had prior radiation therapy to the head and neck. The conventional length shoulder flap could be used to reconstruct defects in the lateral neck region, however, hence the technique of reconstruction of a flap extending from the base of the skull onto the arm was developed. Because of the great length of this flap, its tip extends to any region of the face or neck, even to the contralateral side of the face.

Major Findings:

Twelve patients have thus far had reconstruction of defects of the head and neck region utilizing the extended shoulder flap. Many of these defects would have been virtually impossible to reconstruct utilizing conventional techniques and several extended to the contralateral side of the face.

Significance to Bio-medical Research and Program of the Institute:

The massive defects in the head and neck region resulting from the treatment of massive malignancies referred to the Surgery Branch has heretofore posed a great challenge because of the numerous operations required for the cosmetic and functional rehabilitation of the patient postoperatively. The extended shoulder flap has proved to be a reliable, easily accomplished, and most rapid means of reconstructing such defects.

Proposed Course of Project:

A detailed, illustrated description of the technique of reconstruction utilizing the extended shoulder flap has been prepared. This material was presented at a recent meeting of the Society of Head and Neck Surgeons, and published in a periodical, with a recommendation that it be adopted for use in reconstruction of most defects of the head and neck region not amenable to closure with local tissue. Other uses for this technique of reconstruction are being assessed, e.g., it has been used in combination with the extended deltopectoral flap described in another project in this report in several patients to successfully reconstruct massive full thickness losses of the face virtually impossible to satisfactorily reconstruct with conventional techniques.

Project Description:

Part VIII. Hemipelvectomy: Its role in the treatment of extremity sarcoma.

Objectives:

To develop a technique of hemipelvectomy that minimizes the skill required, the operative time, and blood loss entailed.

Methods Employed:

As a result of the reluctance of surgeons to perform hemipelvectomy when indicated, because of the technical skill required in the execution of the operation lesser procedures which lead to a higher recurrence rate and mortality rate are being employed on a widespread basis. In an effort to improve the survival rate with such patients, we have recently completed a study of the operation in cadavers and living patients and devised a technique that is within the skills of the average general and orthopedic surgeon.

Major Findings:

The conclusion of an assessment of the problem was that the most difficult aspect of hemipelvectomy as conventionally performed was the deep pelvic dissection. We then found that the relatively limited exposure to this anatomically complex and vascular area afforded when the operation is performed with the patient in the supine position is obviated with the patient in the lateral decubitous position. The addition of the Trendelenburg position then afforded additional exposure because of the gravitational shift of the viscera into the upper abdomen. In addition, simultaneous access to the anterior and posterior aspects of the pelvis resulting from this patient position, as well as the perineum when the extremity was elevated, allowed the rapid execution of the operation, particularly in those instances in which uncontrollable bleeding was encountered during the deep pelvic dissection. After studies in cadavers suggested that these concepts should be assessed in living patients, the operation was so utilized with such great success that it was adopted as the standard technique by the Surgery Branch.

Significance to Bio-medical Research and Program of the Institute:

The alarming number of lower extremity malignancies referred to this Institute after recurrence with inadequate operation because of the reluctance of the referring physician to perform hemipelvectomy indicates the need for an easily mastered technique for the operation. The technique developed should have a favorable effect on the surgeon's willingness to perform this operation as the initial procedure, thus giving the patient

the maximum opportunity for cure. In addition, the utilization of this technique by the Surgery Branch has shortened the operative time and the blood loss entailed in the operation, and allowed the safer performance of these operations in relatively poor risk patients.

Proposed Course of the Project:

A detailed and illustrated disclosure of the technique for publication in a surgical periodical is in progress. In addition, a motion picture depicting the advantages of the operation as well as a detailed step by step conduct of the operation in a patient has been completed and submitted to the Film Library of the American College of Surgeons to make it available for distribution to general and orthopedic surgeons.

A. The study of extremity sarcoma.

Objectives:

A review of patients operated on by the Surgery Branch of the National Cancer Institute for sarcomas of bone as well as soft part sarcomas primarily involving the trunk and extremities was undertaken. The questions of survival statistics, incidence of regional node metastases, and the incidence of local recurrence following surgery were of particular interest. Of interest were the results of therapy at the National Cancer Institute as compared to the published results country-wide.

Methods Employed:

Case records of patients operated on by the Surgery Branch of the National Cancer Institute from 1953 until December 30, 1968 were reviewed. Patients studied were those who underwent definitive surgery at this institution.

Major Findings:

Sixty-two patient records were reviewed in detail. The survival statistics for two years and greater demonstrated the following: rhabdomyosarcomas, 3 of 8; liposarcoma, 2 of 4; undifferentiated sarcomas, 1 of 4; fibrosarcomas, 7 of 14; chondrosarcomas, 8 of 11; synovial sarcomas, 1 of 1; mesenchymal sarcomas, 1 of 2; osteogenic sarcomas, 2 of 16; (1 of these 2 was a parosteal sarcoma); leiomyosarcoma, 1 of 1.

This gave an overall survival rate of 26 out of 62 patients.

In this 15 year period there were 21 hemipelvectomies performed and nine interscapular thoracic amputations performed. The mortality from this extensive surgery was minimal, only one patient of these 62 died in the postoperative period and that was a patient who died of renal failure after a combined total pelvic exenteration-hemipelvectomy.

There were four local recurrences, one following radical local excision, two following interscapulo-thoracic amputation, and one following hemi-pelvectomy. These local recurrences were all in the patients who had massive local lesions.

Significance to Bio-medical Research and Program of the Institute:

Survival statistics and local recurrence rates were comparable to published reports. Of particular importance was documentation of the extremely poor outlook for the patient with an osteogenic sarcoma with the survival being at this institution 10% or less. The other common lesion treated here with a poor prognosis was fibrosarcoma with a 50% survival rate greater than 2 years. These data have suggested that we can select patients on the basis of their histological diagnosis and the size of the lesion to represent very poor risk patients and include them in some type of an adjuvant protocol. At present the patients only with metastatic cancer have been treated by immunotherapy but certainly patients with an expected survival of only 10% would be an excellent group to aggressively attack with immunotherapy. Since osteogenic sarcomas presumably do contain a common tumor specific transplantation antigen aggressive immunotherapy seems indicated. In addition, patients with osteogenic sarcomas or large soft part sarcomas would be acceptable for aggressive adjuvant chemo-therapeutic protocol.

Proposed Course:of Project:

The patients who have been operated on since January 1, 1969 will be reviewed to keep the study up to date. This data is being made available to the National Sarcoma Task Force for country-wide evaluation of patients with soft part sarcomas. Further evaluation of rehabilitation of patients who have been amputated is being undertaken to better evaluate patients preoperatively and to assist them in postoperative convalescence. The data is being looked at closely from the standpoint of seeing with which tumors we can avoid radical amputative surgery or particularly in patients whose prognosis is grim.

B. The use of coumadin anticoagulation as an adjunct to surgical treatment of sarcomas.

Objectives:

To anticoagulate patients with primary bone and soft part sarcomas after admission but prior to operative therapy and to achieve a level of approximately 2 to 2-1/2 times control values in terms of prothrombin time. The use of the more sensitive measurement of the level of plasma coagulated factors, namely, the prothrombin-proconvertin (P & P), is utilized in order to control the level of anticoagulation more precisely. This level of anticoagulation is maintained during surgery and also postoperatively. Admission to this study is based on histologically proven sarcoma which is

felt to be capable of cure by amputation, hip disarticulation, hemipelvectomy. Any evidence of metastatic disease or counter indications to anticoagulant therapy excludes the patient.

The patients must be on stable anticoagulation for 72 hours prior to surgery with the prothrombin time 2 to 2-1/2 times control and a P & P 10-25 percent of normal. The initial phases of the study, we have elected not to include patients undergoing hemipelvectomy until we have sufficient experience with the anticoagulation technique. In addition, patients undergoing hemipelvectomy or fore-quarter amputation combined with other major surgical procedures will not be included. However groin or axillary node dissections in combination with amputative surgery does not exclude patients from the study.

Eight patients have been included on the protocol thus far, and have been followed for a little over two years, three have metastatic disease, five do not. All patients had osteogenic sarcomas of the extremity. Three were treated with hip disarticulation, four were treated with above the knee amputation and one had shoulder disarticulation. Three other patients treated with amputation were anticoagulated but the protocol had to be abandoned due to the formation of a hematoma in the stump which required termination of anticoagulation.

Project Description:

Part IX. The surgical treatment of melanoma.

Objectives:

To determine the role of relatively standardized but aggressive surgical approach to malignant melanoma.

Methods Employed:

All lesions are excised with a minimum 4 cm. margin of grossly normal appearing skin. Seldom is closure obtained without skin grafting. Deep subcutaneous and fascial excision encompasses the en bloc primary resection. In continuity lymph node draining resection is carried out if feasible. Bilateral lymph node drainage sites are removed in continuity when midline lesions are encountered. Amputative surgery is not ordinarily indicated or carried out except in finger and toe lesions. Distal extremity melanomas are not necessarily resected in continuity with the groin or axilla. Radical groin dissection is no longer carried out in continuity with the superficial groin unless the superficial groin is confirmed to be positive by frozen section. It is now felt that the radical groin dissection is best carried out by intra-abdominal approach at a second stage thus giving less postoperative morbidity in the form of distressing leg edema.

Major Findings:

This is an ongoing long-term program treating a very distressing type of cancer. The local recurrence rate is less than 7%. Primary cause of an overall long-term mortality, approaching 65%, is due to failure to be able to control distant metastatic disease. Studies are now underway to more adequately evaluate these patients preoperatively as well as attempt to approach their malignant disease with intralymphatic radioactive isotopes.

Significance to Bio-medical Research and Program of the Institute:

This very bizarre-behaving tumor is one of the most poorly understood of the cancers. The tumor from each of these patients is placed into several experimental laboratory protocols. Attempts to develop immunological responses in these patients are being carried out with the use of induced BCG vaccine hypersensitivity.

Proposed Course of Project:

A continued ongoing long-term program with close cooperative efforts in a group of multi-faceted laboratory studies. Patients developing recurrent or metastatic disease will be considered for experimental immunotherapy. 225 patient charts are now being reviewed for determination of the following questions:

1) What influence does the anatomic site of malignant melanoma have on subsequent survival following therapy?

2) What influence does sex have on survival following therapy?

3) Has prophylactic regional node dissection proved beneficial in the treatment of malignant melanoma?

4) Has therapeutic node dissection proved to be beneficial?

5) What factors relating to the primary lesion are important determitants of survival (histology, depth of invasion, size of lesion, etc.)?

6) To what extent has regional node involvement with melanoma influenced survival?

7) Have other forms of therapy utilized in the Surgery Branch provided significant benefit to patients?

8) What complications have followed surgical procedures? To what extent are they seen?

9) Are these particular groups of patients worthy of special scrutiny because of unusual circumstances relating to the course of the disease (e.g., familial melanoma, disease associated with melanoma, prepubertal melanoma, multiple primary melanomas, etc.)?

Project Description:

Part X. Vascular complications of radical cancer surgery of the pelvis and groin.

Objectives:

To review major vascular complications arising during the operative treatment of cancer by radical pelvic or groin surgery, and to derive some principles to be applied in the future for similar situations.

Methods Employed:

A series of cases in which major vascular complications occurred have been critically reviewed and literature regarding these sort of problems has been surveyed. Followup studies on the patients involved have been carried out insofar as availability of patient material permits.

Major Findings:

Ten patients with major vascular complications were reviewed. The conclusions derived from this review were several. The first was that an aggressive approach to these vascular problems is usually the most satisfactory approach. The second is that the early use of contrast angiography, (either venous or arterial) in the assessment of the disease is an important adjunct to solution of the particular problem. The third conclusion is that a major source of post-exenterative venous complications is damage to major vein walls at the time of surgery. It is suggested that in such instances prophylactic caval plication be considered at the time of total pelvic exenteration. The fourth is that in the late followup of patients with leg edema following radical pelvic or groin surgery, benefit may ensue by performing appropriate angiographic studies and vascular reconstructive procedures as indicated thereby.

Significance to Bio-medical Research and Program of the Institute:

As a greater number of people are undergoing radical cancer surgery in the pelvis or groin this is hoped that the data obtained from these studies will lead to a decreasing incidence of complications and a more satisfactory program for treating them when they do arise. The vascular complications of this particular kind of surgery have not been seriously scrutinized in the past and this assessment provides helpful guidelines for further, more detailed evaluation of this problem in this type of patient.

Proposed Course of Project:

Publication of data already obtained. Ongoing surveillance of case material with appropriate studies to substantiate in a prospective fashion the conclusions thus far reached from the retrospective study.

Project Description:

Part XI. Evaluation of pulmonary resection for metastatic disease in patients with apparent control of primary lesion.

Objectives:

To evaluate the potential benefit of pulmonary resection for metastatic disease in patients with controlled primary tumor, and to assess the indications for operation in this group of patients.

Methods Employed:

This was a retrospective study of all the patients having undergone pulmonary resection for metastatic disease by the Surgery Branch, NCI, since its inception. Some twenty patients have undergone such procedures, a few bilaterally. These cases were thoroughly reviewed, both clinically and radiographically. Several significant items of information were noted. Among these were time from control of primary to first detection of pulmonary metastatic disease, correlation between radiographic extent of disease and anatomic extent of disease as determined either at operation or by the pathologist, and survival time following resection.

Major Findings:

The major conclusions from this review are several. First, ordinary PA and lateral chest radiography is not the most appropriate means for assessing the presence of metastatic disease since full chest tomography is far more effective in this regard. The second conclusion is that radiographic evidence universally tends to underestimate the extent of disease. Lesions were identifiable at operation and pathologically which were too small to demonstrate radiographically. Thirdly, although the followup is incomplete on some of these patients, when there is a great discrepancy between the amount of tumor judged present by full chest tomography and the number of deposits found at operation, it is generally unlikely that multiple resections for multiple lesions will lead to a successful outcome. The optimal cases are those in which there are few lesions present and these have all been seen on x-ray. It is also suggested that (1) the rate of development of lesions following control of primary and (2) the growth rate of these lesions as judged by serial tomography will prove to be correlated with the outcome of surgery.

Significance to Bio-medical Research and Program of the Institute:

Both significant palliation and long-term curative benefits have been achieved in a number of patients by pulmonary resection for metastatic disease. It is important to attempt to clearly delineate which patients can be expected to benefit from this type of surgery, so that the number of unsuccessful operations be reduced to the minimum.

Proposed Course of Project:

Publication of data. Ongoing surveillance of clinical material as it presents to the Surgery Branch with this problem, in order to assess prospectively the tentative conclusions from this retrospective study.

Project Description:

Part XII. Significance of tumor doubling time in evaluating operability in pulmonary metastatic disease.

Objectives:

The usual criteria for determining operability in patients with pulmonary metastatic disease have varied and results have been disappointing.

Methods Employed:

The tumor doubling time (TDT) was measured in 113 patients with pulmonary metastatic disease. The TDT was compared to the onset of the metastases, duration, all-type multiplicity of the disease and survival.

Major Findings:

It was found that there was little correlation between survival and the usual criteria used in evaluating patients for resection of metastatic disease. However, there was a good correlation between TDT and survival in both treated and untreated patients. Patients with tumor doubling times of greater than 40 days were found to have unusually good results following resection of their pulmonary metastatic nodules.

Significance to Bio-medical Research and Program of the Institute:

A more uniform and precise method for determining prognosis and changes for survival have been determined in patients with metastatic pulmonary disease.

Proposed Course of Project:

We plan to continue to evaluate an aggressive program of resection of pulmonary metastatic lesions and correlate the tumor doubling time with the results of pulmonary resection in these patients. Hopefully, this will permit us to eventually predict the advisability of pulmonary resection in patients with metastatic disease.

Publications:

Jacobs, J.B., Chretien, P.B., Sugarbaker, E.V., and Doppman, J.L.: Arteriographic discovery of a small contralateral carotid body tumor. Radiology 93: 837-838, 1969.

Chretien, P.B., Ketcham, A.S., Hoye, R.C., and Gertner, H.R.: Extended shoulder flap and its use in reconstruction of defects of the head and neck. Amer. J. Surg. 118: 752-755, 1969.

Ketcham, A.S.: Role of surgery in advanced cancer. U. S. Medicine 4: 1 & 41, 1968.

DeKernion, J.B., Fraley, E.E., Ketcham, A.S., and Paulson, D.F.: Treatment of late complications of ileal conduit. J. Urology 102: 320-324, 1969.

DeKernion, J.B., Ketcham, A.S., and Swerdlow, H.: An improved glass tracheostomy tube. Amer. J. Surg. 117: 759-760, 1969.

Hamner, J.E., and Ketcham, A.S.: Cherubism -- An analysis of treatment. Cancer 23: 1133-1143, 1969.

Harrah, J.D., Holmes, E.C., Paulson, D.F., and Ketcham, A.S.: Serum enzyme alterations after extensive surgical procedures. Ann. Surg. 169: 300-304, 1969.

Ketcham, A.S., Hoye, R.C., and Brace, K.: Irradiation 24 hours preoperative. Amer. J. Surg. 118: 691-696, 1969.

Swerdlow, H., Ketcham, A.S., and DeKernion, J.: Tracheostomy prosthesis. J. Pros. Dent. 22: 84-87, 1969.

Zegarelli, D.D., Ketcham, A.S.: Maintaining the oral and general health of the oral cancer patient. Part I. Cancer 19: 168-184, 1969.

Ketcham, A.S., Hoye, R.C., Pilch, Y.H.: Delayed intestinal obstruction following surgery for cancer. Cancer 25: 406-410, 1970.

Stevens, D.P., Barker, L.F., Ketcham, A.S., and Meyer, H.M., Jr.: Asymptomatic cytomegalovirus infection following blood transfusion in tumor surgery. J. Amer. Med. Asso. 211: 1341-1344, 1970.

Ketcham, A.S., Chretien, P.B., and Pilch, Y.H.: Sarcomas of the head and neck. Symposium on Cancer of the Head and Neck, J.C. Garsford, (ed.) C.V. Mosby, St. Louis, 1969, pp. 337-342.

Ketcham, A.S., Hammond, W.G., Chretien, P.B., and Van Buren, J.M.: Treatment of advanced cancer of the ethmoid sinuses in disorders of the skull base region, Ed. Hamberger and Wersall, Stockholm, Almquist and Wiksell, 1969, pp. 327-344.

Jacobs, J.B., Hammond, W.G., Doppman, J.L.: Arteriographic localization of suprahepatic abscesses. Radiology 93: 1299-1300, 1969.

Harbert, J.C., Fraley, E.E., and Deckers, P.J.: Alterations in the radioisotope renogram pattern after urinary bladder distention. J.A.M.A. 270: 810-811, 1970.

Ketcham, A.S., Hoye, R.C., Pilch, Y.H., and Morton, D.L.: Delayed intestinal obstruction following treatment for cancer. Cancer 25: 406-410, 1970.

Stevens, D.P., Barker, L.F., Ketcham, A.S., and Meyer, H.M., Jr.: Asymptomatic cytomegalovirus infection following blood transfusion in tumor surgery. J.A.M.A.211: 1341-1344, 1970.

Chretien, P.B., Milam, J.D., Foote, F.W., and Miller, T.R.: Embryonal adenocarcinomas (a type of malignant teratoma) of the sacrococcygeal region: Clinical and pathological aspects of 21 cases. Cancer 26: 522-535, 1970.

Hoye, R.C., Ketcham, A.S., and Henkin, R.I.: Hyposmia after paranasal sinus exenteration or laryngectomy. Amer. J. Surg. 120: 485-491, 1970.

Ketcham, A.S.: Modern trends in the prevention of cancer recurrence. In F. Saegesser and J. Pettavel (eds.): Surgical Oncology, Lausanne, Switzerland, Hans Huber Publishers, 1970, pp. 183-211.

Ketcham, A.S., Deckers, P.J., Sugarbaker, E.V., Hoye, R.C., Thomas, L.B., and Smith, R.R.: Pelvic exenteration for carcinoma of the uterine cervix. A 15-year experience. Cancer 26: 513-521, 1970.

Neel, H.B., III, Ketcham, A.S., Hammond, W.G., and Joseph, W.L.: Vascular complications following cancer surgery of the pelvis and groin. Cancer 26: 669-679, 1970.

Chretien, P.B., Carpenter, D.F., White, N.S., Harrah, J.D., and Lighbody, P.M.: Squamous carcinoma arising in a dentigerous cyst. Oral Surg. 30: 809-816, 1970.

Doppman, J.L., and Chretien, P.B.: Visceral pelvic venography in carcinoma of the cervix. Radiology 98: 405-410, 1971.

Joseph, W.L., Morton, D.L., and Adkins, P.C.: Prognostic significance of tumor doubling time in evaluating operability in pulmonary metastatic disease. J. Thoracic Cardiovas. Surg. 61: 23-32, 1971.

Ramming, K.P., Pilch, Y.H., Powell, R.D., Jr., and Ketcham, A.S.: Primary carcinoma in an artificial vagina. Amer. J. of Surg. 120: 108-112, 1970.

Ketcham, A.S.: Newer physical modalities in the treatment of cancer. Proc. Sixth National Cancer Conference, Philadelphia, J.B. Lippincott, Co., 1970, pp. 327-330.

Articles in Press:

Chretien, P.B., Ketcham, A.S., Hoye, R.C., and Sample, W.F.: Axillary dissection with preservation of the pectoralis major muscle. Ann. Surg. in press.

Sugarbaker, E.V., Chretien, P.B., and Jacobs, J.B.: Bilateral familial carotid body tumors: Report of a patient with an occult contralateral tumor and postoperative hypertension. Ann. Surg. in press.

Neel, H.B., Hammond, W.G., and Joseph, W.L.: Upper extremity thrombophlebitis with recurrent pulmonary embolus. Minn. Med., in press.

Joseph, W.L., and Morton, D.L.: Variation in tumor doubling time in patients with pulmonary metastatic disease. J. Surg. Oncol. in press.

Deckers, P.J., Ketcham, A.S., Sugarbaker, E.V., Hoye, R.C., and Thomas, L.B.: Pelvic exenteration for primary carcinoma of the uterine cervix. Obstet. Gynec., in press.

Futrell, J.W., Krueger, G.R., Sample, W.F., Chretien, P.B., and Ketcham, A.S.: Multiple primary sweat gland carcinomas. Cancer, in press.

Serial No. NCI-3805

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Experimental Studies of Mechanisms of Metastases

Previous Serial Number: Same

Principal Investigators: A.S. Ketcham, M.D. and E.V. Sugarbaker, M.D.

Other Investigators: W.G. Hammond, M.D., R.C. Hoye, M.D., P.B. Chretien, M.D., J.J. Ryan, M.D., P.J. Deckers, M.D., A.M. Cohen, M.D., H.B. Neel, III, M.D., and Hilda Wexler

Cooperating Units: Pathologic Anatomy Branch, NCI

Man Years: (July 1, 1970 through June 30, 1971)

Total:	3 1/2
Professional:	1
Other:	2 1/2

Project Description:

Part I.

A. Bioassay of circulating murine tumor cells: A comparison between intravenous and intramuscular inoculation on tumor incidence.

B. The fate of circulating methylcholanthrene tumor cells in mice with tumor specific immunity.

C. The effect of small numbers of MCA tumor cells inoculated I.V. and I.M. in immunized and non-immunized mice.

D. The effect of splenectomy on the survival of circulating tumor cells.

E. The effect of the methods of preparation of single cell suspensions of tumor cells on cell survival in vivo.

F. The effect of prolonged anticoagulation on spontaneous tumor metastases.

Part II.

A. The stimulation of dormant cancer cell growth by hepatic trauma: A study of dormant cancer cells with the Walker 256 carcinoma in the Sprague-Dawley rat.

B. Effects of the adrenal medullary response and exogenous epinephrine in enhancing metastatic spread of tumor.

C. Facilitation of metastasis formation with the Walker 256 tumor in Sprague-Dawley rats with hydrocortisone or cyclophosphamide.

D. Differences in immunogenicity between primary tumors and their metastases.

E. The experimental study of metastases.

1. An attempt to study the process of metastases in vitro using organ explantation techniques.

2. The study of circulating tumor cells by the formation of pulmonary emboli tumor growths in a secondary host.

3. The shedding of viable tumor cells by pulmonary metastases.

F. Immunogenicity of metastases: The role of concomitant immunity in the immunoselection of metastases.

G. The influence of the treatment of methylcholanthrene-induced sarcoma cells with enzymes on tumor immunogenicity.

H. Significance of concomitant immunity as related to the immunologic aspects of metastasis formation.

I. Immunity of the autogenous host to viable tumor cells shed from pulmonary metastases.

Project Description:

Part I.

A. Bioassay of circulating murine tumor cells: A comparison between intravenous and intramuscular inoculation on tumor incidence.

Objectives:

To compare the relative tumor incidence after I.V. and I.M. inoculations of tumor cells in mice.

Methods Employed:

Three host tumor systems were studied: The 89th transplant generation of a methylcholanthrene sarcoma in C57BL6N mice, the 66th generation of a spontaneous mammary adenocarcinoma in C3H/HeN mice, and the S-91 melanoma in CDF1 mice. Pooled blood was aspirated from the right heart of several tumor bearing donors that also had lung metastases. One milliliter was injected into the tail vein of a normal syngeneic recipient, and 1 ml. was injected into the leg muscle of another normal mouse. Pulmonary embolic tumor growths were detected by India ink differential staining for the fibrosarcoma and adenocarcinoma. Melanoma implants were easily identified macroscopically because of their black pigmentation.

Major Findings:

In two of the three tumor groups studied, the I.V. method was more sensitive than the I.M. method in detecting the presence of circulating tumor cells. For CDF1 mice, in 13 pairs the I.V. method detected circulating tumor cells when the I.M. did not. In contrast, in only four pairs the I.V. method failed to detect cells when the I.M. did. For C3H/HeN mice, in six pairs the I.V. method detected circulating tumor cells when the I.M. did not. In contrast, in only two pairs the I.V. method failed to detect cells when the I.M. method did. For C57 mice, circulating tumor cells were similarly detected in both the I.V. and I.M. methods.

Significance to Bio-medical Research and Program of the Institute:

The increased number of pulmonary tumors produced after I.V. inocula of tumor cells in comparison with the numbers of tumors produced with I.M. inoculation of the same tumor cell dose indicates the operation of factors that favor the survival and propagation of tumor cells in the circulating blood in comparison to their survival in extravascular depots. These factors may also be operative in humans with circulating tumor cells shed from a primary malignancy. An investigation and definition of the factors determining the results obtained in this experiment, therefore, may aid in the elucidation of factors that influence the development of blood-borne metastases in cancer patients.

Proposed Course:

A systematic investigation of the factors determining the selection survival of tumor cells in the circulating blood is in progress.

Project Description:

B. The fate of circulating methylcholanthrene tumor cells in mice with tumor specific immunity.

Objectives:

To assess the effect of immunity on the survival of circulating tumor cells.

Methods Employed:

One host system was studied. An immunogenic methylcholanthrene tumor was injected into syngeneic mice, and the resulting tumor growth was then removed. The production of tumor specific immunity to these mice was shown by I.M. challenge with cell suspensions of the tumor. The effect of immunization to the tumor on circulating tumor cells was assessed by determining the number of lung tumors that resulted from the I.V. injection of cell suspensions of the tumor and blood from donor mice that had circulating tumor cells.

Major Findings:

Syngeneic mice were immunized to a methylcholanthrene-induced tumor and immunity was demonstrated by the failure of cell suspensions of the tumor to grow when inoculated intramuscularly. Identical cell suspensions inoculated intravenously, however, produced pulmonary tumors in all immunized animals. Intravenous inoculations of blood containing circulating tumor cells obtained from donor mice bearing the same tumor produced identical numbers of pulmonary tumors in immunized and unimmunized animals.

Significance to Bio-medical Research and Program of the Institute:

In this study, immune resistance to tumor growth as demonstrated by the failure of I.M. inocula to grow in animals previously exposed to the tumor was not reflected in a similarly heightened resistance to tumor growth where the tumor cells were inoculated I.V. In this tumor system, therefore, the ability of the tumor cells that leave the primary to survive and give rise to secondary growths is dramatically increased if they gain entrance into the circulatory system. This paradoxical increased survival and production of pulmonary embolic tumor by circulating tumor cells grows in hosts with tumor specific immunity may have relevance to the development of pulmonary metastasis in patients who have demonstrated specific immunity to their previously excised primary tumor. The presence of factors determining the results in this experiment in such patients would facilitate the survival of tumor cells that entered the circulatory system prior to the excision of their primary tumor.

Proposed Course of Project:

Manuscript accepted for publication in Cancer.

Project Description:

C. The effect of small numbers of MCA tumor cells inoculated I.V. and I.M. in immunized and non-immunized mice.

Objectives:

To assess and compare the survival of various tumor cell doses when inoculated I.V. and I.M. in immunized and non-immunized mice.

Methods Employed:

One host system was studied. Immunogenic methylcholanthrene tumor was injected into syngeneic mice, and the resulting tumor growth was then removed. Cytosieve cell inocula of 1000, 500, 100 and 50 cells were then prepared from this tumor. Normal recipient mice were inoculated either I.V. in the tail vein or I.M. in the right leg muscle with these various tumor cell doses. The production of tumor specific immunity to these mice was shown by I.M. challenge with a 1000 cell suspension of the tumor.

Major Findings:

One thousand tumor cells produced tumors at the site of I.M. inoculation in 90% of untreated mice and in only 10% of the mice that previously had amputation of the same tumor. The same tumor cell doses, when inoculated I.V. produced pulmonary tumors in 90% of both the control and the immunized mice. As the tumor cell inocula decreased in size there was a progressive decrease in the number of animals that developed tumors at the site of I.M. inoculation. With 50 cells, neither the controls nor the immunized mice developed tumors at the site of I.M. inoculation. The same dose inoculated I.V., however, produced pulmonary tumors in 30% of the untreated animals and 64% of the immunized animals.

Significance to Bio-medical Research and Program of the Institute:

The results support earlier work done with the same immunogeneic tumor. Specific immune inhibition of tumor growth as assessed by I.M. inoculation was not reflected in the destruction of cell suspensions of the tumor when injected into the circulatory system. The increased survival of small numbers of tumor cells when given I.V. in immune animals as compared to controls shows that tumor specific immunity in this experimental system actually favors the survival of tumor cells in the circulatory blood. Experiments that further define the factors that enhance the survival of circulating tumor cells in immunogenic animals should aid in the understanding of the factors that determine the development of blood-borne metastasis in patients with certain malignancies.

Proposed Course of Project:

Further experiments are in progress that are designed to reveal those functions which promote the survival of tumor cells when introduced into the circulatory system of mice that have specific immunity to the tumor.

Project Description:

D. The effect of splenectomy on the survival of circulating tumor cells.

Objectives:

To evaluate the effect of splenectomy on the development of pulmonary embolic tumors from tumor cells inoculated I.V. in C57BL6N mice.

Methods Employed:

One host tumor system was studied. The eleventh generation of an immunogeneic tumor (MCA) was used. Immunogenicity was demonstrated by the injection of the tumor I.M. into mice that had previous amputation of right leg tumors when they were 1 cm. in size. Tumor cell suspensions containing 100, 600, and 1200 cells were injected I.M. and I.V. into previously splenectomized and control mice. After three weeks all animals were sacrificed and the number of pulmonary embolic tumors was determined using the India ink method.

Major Findings:

As in previous experiments with this tumor system, when 1000 tumor cells were inoculated I.V. significantly more animals developed pulmonary tumor than did animals developing leg tumors at the site of I.M. inoculation of 1000 tumor cells. Splenectomy reduced both the number of I.M. tumor and the number of pulmonary embolic tumors produced. The differences between the numbers of pulmonary embolic tumors in splenectomized and non-splenectomized mice increased with progressively smaller tumor cell inocula.

Significance to Bio-medical Research and Program of the Institute:

These results show that the spleen is implicated in the increased survival of circulating tumor cells injected I.V. that has been previously demonstrated in this laboratory. This supports the experimental findings in other laboratories that the spleen is a primary source of factors that enhance tumor growth. The mounting clinical evidence that patients with certain tumor have improved survival after splenectomy may possibly be related to factors operative in this experimental tumor system.

Proposed Course of Project:

Further investigation of the role of the spleen in the selective survival of I.V. tumor cell are in progress. At present, the effect of splenectomy on the previously demonstrated increased survival of I.V. tumor cells in animals previously immunized to the tumor is being studied.

Project Description:

E. The effect of the methods of preparation of single cell suspensions of tumor cells on cell survival in vivo.

Objectives:

To compare the effect of enzymatic (pronase and trypsin) and mechanical (cytosieve) methods of producing single cell suspensions of solid tumors by assaying the tumor mixture resulting from the suspensions when inoculated I.V. and I.M.

Methods Employed:

One tumor system was studied. Single cell suspensions of a solid MCA induced tumor were prepared, using pronase, trypsin and mechanical (cytosieve) disruption. The percent viable cells in each suspension was determined by trypan blue exclusion. The cell suspensions were adjusted with Hank's balanced salt solution to contain 1000 cells in .5 ml. The suspensions were inoculated I.V. and I.M. into C57BL6N mice. All animals were sacrificed when animals in any group were found dead. The number of pulmonary tumors and leg tumors were determined.

The effect on tumor incidence of the cellular debris resulting from a cytosieve preparation was studied separately. A single cell suspension prepared using the cytosieve method was divided into two parts. Half of the suspension was centrifuged and the supernatant debris discarded. Both halves were adjusted to contain 1000 cells in .5 ml. and inoculated I.V. and I.M. into C57BL6N mice. The animals were sacrificed three weeks later and the incidence of lung and leg tumors determined.

Major Findings:

The single cell suspension with the cytosieve method produced tumors in all animals. Pronase prepared cells produced lung tumor in 47% and leg tumors in 73% of animals and trypsin produced lung tumors in 60% and leg tumors in 49% of animals.

Removal of the cellular debris did not alter the incidence of tumors produced by the single cell suspension prepared with the cytosieve technique.

Significance to Bio-medical Research and Program of the Institute:

One of the essential techniques in the study of experimental tumors is the preparation of single cell suspensions. The technique that least alters the characteristics is desired. Controversy continues concerning the most ideal method of single cell suspension preparations. The objection to mechanical suspension is the effect on cell viability and the possible effect of cellular debris on transplantability of the cells. Enzymatic separation methods are suspect of altering cell characteristics by an effect on the cell wall. In this experiment, the results with mechanical disruption show that the cellular debris does not alter tumor development, and a greater number of tumor, both I.M. and I.V. were produced than with either the pronase or trypsin preparation.

Proposed Course of Project:

This data is being prepared for publication.

Project Description:

F. The effect of prolonged anticoagulation on spontaneous tumor metastases.

Objectives:

1. To evaluate the effect of long-term anticoagulation on the size of a primary tumor and its spontaneous metastases in an isologous host system.

2. To observe morphology and fibrin content of a tumor growing in an anticoagulated animal. The coagulation mechanism is believed to play a role in the initial adherence of embolic tumor cells and subsequent development of distant metastases. Transient anticoagulation decreases the number of pulmonary metastases in animals given tumor cells intravenously. This study evaluates the effect of long-term anticoagulation on the size of a primary tumor and its spontaneous metastases in an isologous system.

Methods Employed:

Two host-tumor systems were studied: The methylcholanthrene sarcoma T241 of Lewis in C57 Black 6N mice and the spontaneous mammary adenocarcinoma in C3H/HeN mice. Following tumor inoculation one-half of the mice in each system were begun on oral sodium warfarin at a constant pre-determined dosage. At five intervals equal numbers of control and anticoagulated mice were sacrificed after prothrombim time determinations. By India ink differential staining of tumor and lung tissue, metastases were detected macroscopically.

Major Findings:

Prothrombin times of the mice were maintained continuously in the range of 2 to 3 times normal and the mice free of any hemorrhagic complications. In each of the five intervals of comparison in both host-tumor systems at least three time as many control as anticoagulated mice had metastases. For the C57 mice a cumulative total of 73% of controls and 15% of anticoagulated mice had metastases and for the C3H system, 62% of controls and 15% of these anticoagulated.

Significance to Bio-medical Research and Program of the Institute:

The results support the earlier work done with an early generation of methylcholanthrene induced fibrosarcoma. In all three systems long-term anticoagulation has significantly reduced the incidence of spontaneous metastases and significantly increased the number of individuals free of metastatic disease. The more recent studies show this effect of anticoagulation will persist when the level of anticoagulation is maintained constantly in a safe range free of any hemorrhagic complications. This further attests to the importance of the coagulation mechanism in the intravascular metastatic spread of tumors.

Proposed Course of Project:

The use of anticoagulation as an adjunct to standard therapy with surgery or irradiation is being evaluated.

Project Description:

Part II.

A. The stimulation of dormant cancer cell growth by hepatic trauma: A study of dormant cancer cells with the Walker 256 carcinoma in the Sprague-Dawley rat.

Objectives:

To provide a model for the study of dormant cancer cells.

Methods Employed:

A critical aspect of the problem of cancer metastases revolves about the dormant cancer cell. Clinically it has been repeatedly documented that patients many years after resection of a primary lesion and apparent cures suddenly succumb to widely disseminated and rampantly progressive metastatic growth after minor trauma or major surgery. An experimental model for dormant cancer cells was reported by Bernard Fisher in Pittsburgh in 1959. Two weeks after injection of Walker 256 cancer cells into the portal vein of rats, liver massage produced gross metastases which otherwise would not develop.

A study of dormant cancer cells was undertaken using this same model. The first step listed below was to demonstrate this phenomenon repeatedly and verify these experimental results. A cytosieve preparation of solid Walker 256 tumor was prepared under sterile conditions. Five thousand, 25,000, and 125,000 viable tumor cells as proven by trypan blue exclusion were injected into the portal vein of 150 gm. Sprague-Dawley rats at laparotomy. The abdomen was closed with interrupted through and through sutures of silk. At 4, 6, and 8 weeks of post-injection, the animals were opened and livers grossly negative for metastases at that time were manipulated. All surrounding attachments of liver were taken down, the liver compressed, and in essence a sham-hepatectomy performed. These animals were again closed. At 4 week intervals thereafter the animals were re-massaged until tumor developed or until 8 massages had transpired. The number of animals in control groups having metastases and the number of dormant cells stimulated to clinical growth were tabulated.

Major Findings:

With the 5,000 cell injections no animals developed metastases. With repeated massage 64% of 3 series (40 to 45 animals per series) developed static metastases - biopsy proven. With the 25,000 and 125,000 cell control animals about 10% developed hepatic metastases in each group. With serial massage a total of about 70% developed hepatic metastases.

Significance to Bio-medical Research and the Program of the Institute:

A study which potentially could delineate factors governing the resurgent growth of dormant cancer cells would be of utmost importance in the control of clinical disease. Such late recurrences occurring 2 to 15 years post-operatively account for a major proportion of surgical failures.

Planned Course of the Project:

A benzpyrene-induced tumor in an inbred strain of Marshall rats will be used to try to replicate these experimental results. This system lends itself to immunologic studies. It would be of a great interest to know what the immunologic status of each animal is toward these dormant tumor cells. In the usual host-tumor system with tumor specific antigenicity demonstrated, such small numbers of cells should be rejected.

A study in organ explanted tissue culture is also planned. Needle biopsies of rat livers containing dormant cancer cells will be planted into tissue culture medium and various manipulations performed to try to encourage their growth in tissue culture. It is thought that this in vitro system would allow manipulation of the mileiu of dormant cancer cells and perhaps definitive results can be obtained outside the complexity of the total organism.

A further extension of this study involved studies of the immunogenicity of the benzpyrene sarcoma and also of the Walker 256 tumor. It was shown that the benzpyrene sarcoma was highly and specifically immunogenic in the inbred Fisher rats. The Walker 256 tumor, even in the outbred Sprague-Dawley animals, could not be demonstrated to carry any degree of immunogenicity. However, these studies were not carried to extreme degrees, although a full range of tumor cell challenge doses of animals immunized with partial tumor growth were undertaken. It was concluded that the Walker 256 tumor at best was much less immunogenic than the benzpyrene tumor.

It was, therefore, hypothesized that the ability of a tumor to establish itself in the dormant state might be related to the immunogenicity of the tumor cell itself. The highly immunogenic tumor cells were destroyed by host defenses or overcame them. Weakly immunogenic tumors could apparently co-exist with host defenses in a non-dividing state until recalled by a surgical stress.

Project Description:

B. Effects of the adrenal medullary response and exogenous epinephrine in enhancing metastatic spread of tumor.

Objectives:

1. To determine if exogenous epinephrine enhances the tumor take.

2. To determine the dose response relationship of epinephrine effect of enhancing tumor take.

3. To compare various doses of epinephrine combined with and also without corticosteroids and a regulated systemic stress (right nephrectomy) in enhancing tumor take.

Methods Employed:

We have demonstrated that experimental metastases formation is enhanced by a simultaneous surgical trauma. The mechanism through which such occurs has been obscure. Hydrocortisone in supraphysiologic doses mimics the effects of trauma but its immunosuppressant effects are hard to separate from the effects of hydrocortisone in physiologic doses. No investigations to date have observed the effects of chemical means and specifically epinephrine on metastasis formation, especially if given in physiologic doses. Supraphysiologic and physiologic doses of epinephrine were given to groups of syngeneic C57 black female mice prior to the intravenous infusion of T241 sarcoma cell suspensions prepared with pronase. Control animals were either untreated, receiving only saline placebo, or right nephrectomy as a standard surgical stress. Initial experiments with supraphysiologic doses of epinephrine

demonstrated a marked enhancement of metastasis formation with the drug. The nephrectomized animals had an increase of borderline statistical significance, but on repeat studies, these values were confirmed at $P<.05$ levels. Studies with physiologic doses of epinephrine were performed and again a marked enhancement of metastasis formation observed. Studies were then carried out before and after tumor cell infusion, and it was found that when the drug was administered after tumor cell infusion, this effect in enhancing metastasis was no longer observed. These data suggest that the effects of a surgical stress can be mimicked by administration of exogenous epinephrine in physiologic doses. It has been established in other experiments that adrenaline triggers blood hypercoagulability, presumably as part of the stress reaction as described by Seyle. This may be the mechanism through which a surgical stress or trauma enhances metastasis formation.

Project Description:

C. Facilitation of metastasis formation with the Walker 256 tumor in Sprague-Dawley rats with hydrocortisone or cyclophosphamide.

Objectives:

To determine the metastatic pattern from spontaneous metastases in the Walker 256 carcinoma transplanted to the leg.

Methods Employed:

One cc. of tumor brei was injected into the right hind leg of Sprague-Dawley rats (150 g.). These animals were then followed to death and the lungs and all body organs examined.

Major Findings:

The Walker 256 carcinoma originated in the Walker laboratory in Baltimore at Johns Hopkins about 1928. The metastatic pattern of this tumor has been described in many literature publications and by report it infrequently metastasizes and when it does so, it does so primarily via the regional lymph nodes. This had not been our observation and we attempted to study spontaneous metastases from induced leg tumors in individually-housed animals. Ninety-eight percent of animals developed diffuse pulmonary metastases. Three percent of animals developed regional lymph node metastases. No other organ involvement was noted. Animals survived 4-7 weeks post-inoculation.

Significance to Bio-medical Research and Program of the Institute:

This information provides working data for ongoing projects involving the Walker 256 carcinoma in Sprague-Dawley rats.

Proposed Course of Project:

Using spontaneously metastasizing Walker 256 carcinoma, a project is planned which should help to answer the question as to whether or not lesions that have once metastasized have a greater propensity to do so from the metastases themselves. In this project, spontaneous metastases will be excised from the lungs of sacrificed animals, a cell suspension prepared and a given dose injected intravenously in control animals. A like dose of single cells from solid tumor will be injected into control animals. A significantly greater take in the cells prepared from the pulmonary metastases would suggest that these cells have a greater propensity to take after intravenous injection than the cells from the primary tumor.

The role of the immune mechanism in the control of metastasis formation is of particular importance with regard to cancer chemotherapy since most cytostatic drugs are simultaneously immunosuppressive. This study was undertaken to observe the effects of two commonly used chemotherapeutic agents alone or in combination on the pattern of spontaneous metastases from a transplantable tumor. The Walker 256 tumor in Sprague-Dawley rats metastasizes preferentially and specifically to lung and to a lesser extent regional lymph nodes. In our year and a half experience with this tumor, it has never metastasized to other viscera. Animals were treated with hydrocortisone, cyclophosphamide or both drugs. With drug treatment a marked increase in the incidence of visceral metastases occurred. It was hypothesized that this facilitation of metastasis in the Sprague-Dawley rat might be related to depressed immunity to circulating or "dormant" tumor cells.

Project Description:

D. Differences in immunogenicity between primary tumors and their metastases.

Objectives:

To test the hypothesis that a mechanism of immunoselection is operative among those cells shed from the primary tumor into the blood stream and that the less immunogenic cell or cells are those that develop as metastases.

Methods Employed:

Metastases and primary tumor from the same animal are separately transplanted into different groups of animals and the tumor-bearing legs amputated when the tumors reach 1.0 cm. in diameter. Seven to ten days later equal doses of each tumor line are given to control mice and to mice immunized with either the primary or metastatic tumor line. Development of tumors at the site of challenge injection is noted. Comparison between control mice and those previously bearing one of the tumor lines is carried out using a statistical method of evaluation based on computer analysis of slopes and intercepts of

the regression lines. This then gives us three different parameters of differences between the groups of mice each parameter being readily accessible for statistical and biological significance.

Major Findings:

In the four tumor-host systems thus far studied, the pattern of immunogenicity between primary tumor and metastatic tumor has varied depending upon the challenge dose of tumor used and the particular original tumor. In one system, there was enhancement of challenge tumor growth in immunized animals. Present data are not complete, but there is a suggestion that the presence of occult metastases from the first exposure to tumor growth produces an enhancing effect on the growth of the subsequent challenge inoculum.

Significance to Bio-medical Research and Program of the Institute:

As progress is made towards effective immunotherapy for human cancer, it becomes more clear that the major target initially will be metastatic disease. Therefore, it is of importance to know whether immunologic measures directed against primary tumor antigens will be effective against metastatic lesions. The demonstration of enhancement of tumor growth in one area by the presence of occult disease elsewhere is of interest in that it may provide an experimental basis for clinical observation that, in certain patients, there appears to be a rapid spurt in the growth of tumor due to an unknown mechanism. Additionally, this may provide some understanding of the clinical observation that removal of primary tumor or reduction in bulk of tumor at the primary site may occasionally result in regression of metastatic disease. The method of life-table analysis of this kind of data has also added a useful methodologic procedure, since data not thought significant by inspection can be shown to be useful by this means and hence increase the yield of information from the same amount of animal work.

Proposed Course of Project:

More detailed investigation is underway regarding the effect of occult metastases on the growth of tumor at the primary site. Simpler methods for assaying differential immunogenicity are undergoing scrutiny, particularly the delayed cutaneous hypersensitivity reaction to membrane fractions prepared from tumors.

Project Description:

E. The experimental studies of metastases.

1. An attempt to study the process of metastases in vitro using organ explantation techniques.

Objectives:

The general goals of this project have been to study the process of metastasis in vitro. The entire field of metastasis formation is extremely difficult to study because of the complex of variables interacting at the three major phases of the process of metastasis, that is, the processes involved in the release of cells, the selection of metastasizing tumor cells from those in the circulation, and the actual arrest and growth of the metastasis. It had been hoped that this process could be duplicated by infusing tumor cells into organ explant tissue cultures in vitro.

Methods Employed:

Step 1 was to confirm the fact that the organ explants of appropriate tissues survived well in vitro. Newborn C57 mouse tissue was dissected under aseptic techniques in explants of lung, liver, muscle, skin, kidney tissues. Approximately eight 1 mm. cubes of tissue were placed on a Trowell tissue culture grid in the Trowell tissue culture dishes and using 1640 tissue culture medium supplemented with 20% fetal calf serum and in an environment of 5% CO_2, these were allowed to grow. Actually progressive growth never occurred, although viability was maintained in about 90% of explants. Explants were removed first at several days intervals and then at weekly intervals and essentially 80% survival obtained at six weeks as monitored by histologic means. All explants survived well except liver, which in no case survived longer than one week. Progressive deterioration of the cytoarchitecture occurred and eventually just an amorphous debris was obtained with liver explants. Interestingly, the explants grow out onto the fibers of the stainless steel grid and a rather intricate meshwork can be identified.

Major Findings:

With adequate survival of the explants, several types of studies were attempted. First, it was attempted to demonstrate the phenomenon of preferential metastasis in vitro by infusing tumor-cell containing tissue culture media into viable explants. Various concentrations of tumor cells ranging from 10^5 through 10^7 cells per cc. were used. In group A experiments, four different organ explants were placed on the same grid, and in group B experiments, four of the same type of explants were placed on each grid. Tumor cells seemed to adhere selectively to lung tissue only. These studies, however, need to be repeated as, unfortunately, in this latter study, several infections obscured results.

In a second group of experiments, the explant of C57 tumor tissue, that is the seventh generation of a known immunogenic fibrosarcoma induced in C57 mice, was placed on a grid with three different types and also three different explants of the same type. In this situation, as the tissue culture media was sequentially changed, the medium was spun and the pellet counted for the presence of tumor cells. It was demonstrated that viable tumor cells were shed from these tumor explants in vitro, however, they did not "metastasize"

to other explants in vitro. At least no metastasis was observed histologically. It was planned that radio-tracer methods be applied to this technique once the methods have been perfected. Plan for future studies is to repeat the experiments above which gave some suggestive results and also to apply a 95% oxygenated atmosphere in hopes that this will enhance metastasis formation and explant survival.

Proposed Course of Project:

We feel that persistence in perfecting the technical details of such a method is important. The entire field of metastasis is in need of an in vitro system with which to sort out the complex variables which have obstructed progress in this field.

Project Description:

E. 2. The study of circulating tumor cells by the formation of pulmonary emboli tumor growths in a secondary host.

Objectives:

This method was studied in an attempt to better assess the metastatic potential of circulating tumor cells contained in the circulation.

Methods Employed:

Three tumors were used in this study. Tumor inoculum was prepared subcutaneously by the Snell cytosieve technique. This tumor inoculum was then implanted into donor mice. At various intervals after implantation, venous blood was withdrawn from the right side of the heart of these animals and then this blood was injected immediately into the tail vein of an identical syngeneic recipient host; these recipients were then killed 28 days after injection. Pulmonary metastases were identified in both the donor and the recipient mice by using the India ink technique.

Major Findings:

Pulmonary emboli tumor growths were found in some recipient mice of all 3 strains, and these tumors were histologically identical to the primary tumor and pulmonary metastases of the donor mice. The presence of circulating tumor cells in secondary hosts occurs simultaneously with or shortly following the onset of spontaneous metastases in donor mice. This reflects a more accurate assessment of metastatic growth potential for the circulating tumor cells, rather than using cytologic techniques for the same purpose.

Significance to Bio-medical Research and Program of the Institute:

This method of detecting circulating tumor cells in the blood of mice allows some assessment of the metastatic potential of contained pulmonary embolized tumor cells.

Proposed Course of Project:

To compare the sensitivity of this method with other methods in detecting the presence of circulating tumor cells and the ability to quantitate the numbers of cells in the blood of mice.

Project Description:

E. 3. The shedding of viable tumor cells by pulmonary metastases.

Objectives:

To demonstrate that the formation of pulmonary embolic tumor growths in recipient mice identical to the pulmonary metastases in a primary host would be suggestive that pulmonary metastases have metastasized.

Methods Employed:

Three tumor-host systems were studied: The T241 sarcoma of Lewis in C57BL/6N mice, the mammary adenocarcinoma in C3H/HeN mice, and the S-91 melanoma in CDF_1 mice.

Primary tumors were established by the inoculation subcutaneously in the thigh of 0.05 ml. of a uniform cytosieve tumor-cell suspension. Twenty C57BL/6N mice, 30 C3H/HeN, and 25 CDF_1 mice were thus inoculated. The primary tumors were then allowed to grow for a period of time, determined by previous study, in which pulmonary metastases would form in virtually all mice yet the primary tumor would still be completely removable by amputation. This interval between inoculation and amputation was 9 days for the C57BL/6N mice, 13 days for the C3H/HeN, and 21 days for the CDF_1 strain. Amputation was performed with a cutting cautery. A period of time was then allowed for maximal enlargement of the pulmonary metastases, yet avoiding deaths due to them. This interval was four weeks for the C57BL/6N and C3H/HeN mice and six weeks for the CDF_1 strain. These mice then had 0.6 cc. of blood drawn from the left ventricle under direct vision, using a #27 needle and heparinized syringe. This was the maximal quantity which could routinely be obtained. The blood was immediately injected into the tail vein of an identical syngeneic mouse. After a four week interval these recipient mice were sacrificed, and their lungs examined for pulmonary embolic tumor growths. This was done macroscopically using India ink-Feket's solution differential

staining of normal lung tissue and tumor for the C57BL/6N fibrosarcoma and C3H/HeN mammary carcinoma systems. The pulmonary tumor growths of the recipient mice were compared histologically with the primary tumor and pulmonary metastases of the donor mice in all three systems.

Major Findings:

In the primary tumor-bearing donor mice, 100% of both the C57BL/6N and the CDF_1 and 93% of the C3H/HeN mice had pulmonary metastatic lesions at the time pulmonary venous blood was drawn from the left heart. The pulmonary metastatic spread was further quantitated. Among the 20 C57BL/6N donor mice, 18 had metastases too numerous and confluent to permit accurate counting, one had 8 metastatic deposits, and one had 5. In the 30 C3H/HeN donor mice, 8 had lesions too confluent to count, 5 had between 6 and 10 metastases, 15 mice had between 1 and 5 lesions, and 2 were free of pulmonary metastases. All 25 CDF_1 mice had lung metastases too numerous and confluent to count. Thus the great majority of donor mice had a large amount of metastatic tumor in their lungs.

In the recipient mice receiving the tail vein injections, 40% of the C57BL/6N, 20% of the C3H/HeN, and 16% of the CDF_1 mice had pulmonary embolic tumor growths. Of the 8 C57BL/6N mice with pulmonary embolic tumors, 3 had lesions too numerous and confluent to count, 1 had 5 lesions, 1 had 3 lesions, and 3 had 1 lesion each. Among the 6 C3H/HeN mice, 2 had 4 lesions, 2 had 2 lesions, and 2 had 1 lesion each. Of the 4 recipient CDF_1 mice with lesions, 1 had 9 lesions and 3 had 1 lesion each. Thus while many of the recipient mice had a solitary embolic tumor growth, the majority had multiple lesions and in a few mice the lesions were too numerous and confluent to count.

On histological review, the pulmonary embolic tumor growths of the recipient mice were identical to the metastatic and primary tumor growths of the donor mice in all three tumor systems.

Significance to Bio-medical Research and Program of the Institute:

The formation of pulmonary embolic tumor growths in the recipient mice of all three tumor-host systems clearly establishes that pulmonary metastases do shed viable circulating tumor cells. Alternate sources of such cells, other metastatic lesions or residual primary tumor, were evident in none of the donor mice. The fact that circulating tumor cells have not been detectable in the blood beyond 5-6 hours following intravenous injection, makes the primary tumors, removed weeks before, an unlikely source of the tumor cells in the pulmonary venous drainage in the present study.

The formation of metastatic lesions does not necessarily follow from the presence of circulating tumor cells. Clinical studies of circulating tumor cells have found no good correlation between the detection of circulating tumor cells and metastatic spread. However, in the present study, the formation of pulmonary lesions in a syngeneic host clearly establishes the

viability and metastatic potential of the circulating tumor cells. It is equally clear that this metastatic potential might not have found expression by the formation of metastases in the donor mice due to host resistance induced by previous tumor exposure. The methylcholanthrene-induced sarcomas have been shown to be immunogenic and previous exposure protects against a second tumor inoculum. Whether tumor immunity does indeed modify or influence spontaneous metastatic spread is not established. However, with the shedding of viable tumor cells from pulmonary metastatic lesions established and the potential of these cells to form pulmonary tumor growths, similar to metastases, demonstrated it appears probable that pulmonary metastases can metastasize. In a consideration of the management of solitary pulmonary metastases in the patient, suggestions arising from the results of this study must certainly be examined against the obvious host differences involved. The behavior of transplanted tumors in animal hosts may differ importantly from the behavior of autochthonous tumors in patients. Nevertheless, the demonstrated ability of pulmonary metastases to shed viable circulating tumor cells would argue a prolonged period of observation of a pulmonary lesion. Further metastatic spread from such a metastatic deposit would appear distinctly possible.

Proposed Course of Project:

Further experimental studies with additional tumor systems will be carried out. Application to problems in the human will be appropriately investigated.

Project Description:

F. Immunogenicity of metastases: The role of concomitant immunity in the immunoselection of metastases.

Methods Employed:

Using the benzpyrene-induced sarcoma of Fisher rats (a syngeneic system) animals were inoculated with tumor. After tumor grew to metastasizing size as demonstrated by pilot experiments, these animals were amputated. Several weeks later, a group of animals was sacrificed and in one animal, there were three discrete metastases each weighing approximately 1 gram. These were dissected and enzymatic preparations made of each metastatic cell line and also of the primary which had been carried along simultaneously in other animals. Groups of 30 rats were immunized with each of these tumor cell suspensions. The growth characteristics of M0 and M2 did not differ significantly from the primary. M1, however, grew very slowly and these animals were discarded. Tumors were amputated at approximately 1-1/2 to 2 cm. in diameter and one week after amputation, cross-challenges of all groups carried out with all lines of M0, M2, and P. After 60 days observation, the rats challenged respectively with M0, M2 and P showed the following incidence of tumor.

Major Findings:

M0 immune - 0 of 10, 5 of 10, and 1 of 9; M2 immune - 7 of 10, 0 of 10, and 4 of 11; P immune - 10 of 11, 9 of 12, and 0 of 10. Non-immune controls showed 27 of 30, 11 of 30, and 21 of 30. These results show that M0, M2, and P are immunogenic cell lines to P values less than .01. There was no inhibition of M0 or M2 "take", latency, or growth in animals immunized with P. Immunization with M0 or M2 significantly decreased the "take" of P ($P<.05$).

Immunization with M0 did not inhibit "take" or growth of M2 although M2 immunization gave protection against M0 challenge. Identical experiments were repeated in a second transplant of M0 and M2 and the 9th generation of P, comparable immunogenic differences persisted. These data indicate that metastases can be immunogenically distinct from the primary and from each other in the same experimental host. It was hypothesized that concomitant immunity might result in the selection of immunogenically altered cells from the many circulating tumor cells present.

Significance to Bio-medical Research and Program of the Institute:

A significant conclusion was reached in this study, that is, that these metastatic cell lines were immunogenically different from the primary. Current studies to expand these observations are underway. No. 1 - first, cell neutralization studies using spleen cells from animals immunized with three different cell lines are being mixed with the various cell tumor lines and re-inoculated for in vivo assay. These methods have been extensively described by Winn (1964).

Another study is underway to rule out the modulation of the tumor cell immunogenicity by the preferential metastasis to lung. Normal unimmunized animals have been injected intravenously with graded tumor cell doses. It is hoped that discrete metastases can, therefore, be dissected out and the above experiments essentially repeated, except in animals who were non-immunized initially. If concomitant immunity does actually serve as an immunoselective device, then in these animals who were exposed unimmunized, the metastases should be immunogenically identical with the primary.

These studies are also being duplicated using a syngeneic C57 methylcholanthrene-induced mouse sarcoma. We have had some difficulty in obtaining growth of metastatic lines when re-inoculated into recipients. Obviously in the mouse system a first transplant of the metastatic lines must be used since metastases are much too small to immunize a significant number of animals primarily. We have also in the mouse system looked into the ability of an immunized host to reject sarcoma cells with intravenous infusion. Preliminary studies in which animals were challenged 17 days after amputation showed little effect of immunization in decreasing pulmonary metastases. A major problem with this study was that the challenge interval was 17 days and other studies have shown that there is a fall-off of immunity after 10 days. Studies are currently underway, and have been performed, in which

animals were challenged with intravenous tumor cells at 7, 14, and 21 days post-amputation of their immunizing tumor growth and results should be available soon on this study.

If it is demonstrated that these metastases are definitely immunogenically different from the primary tumor, rather broad sweeping implications will be forthcoming for the whole field of immunotherapy.

Project Description:

G. The influence of the treatment of methylcholanthrene-induced sarcoma cells with enzymes on tumor immunogenicity.

Objectives:

A pervading assumption in much of the data which pertains to tumor specific antigens in experimental systems assumes that the enzymatic treatment of solid tumors in order to prepare quantitative inocula does not alter tumor cell immunogenicity.

Methods Employed:

In this group of experiments, animals immunized with mechanical "cytosieve" preparations and enzymatic trypsin or pronase preparations were used to immunize animals. These animals were then cross-challenged with the same types of preparation. Also a tissue culture preparation of the same tumor was passed two generations and used to immunize animals, and, the fourth passage used to cross-challenge all groups.

Major Findings:

Results demonstrated that up through challenge doses in the range of 5×10^5 cells, there was no difference in the immunogenicity of these preparations. However, when challenged with 10^6 cells an emerging pattern occurred. Animals immunized with the cytosieve preparation were less immune to challenge doses of the enzyme preparation than those challenged with the same amount of viable tumor cells prepared mechanically. It was concluded that for the usual experimental situations that enzymes have no real discernible effect on tumor cell immunogenicity. However, with the extremes of challenge dose, there is a small difference which should be considered an experimental design.

Project Description:

H. Significance of concomitant immunity as related to the immunologic aspects of metastasis formation.

Objectives:

To ascertain the frequency and demonstrability of concomitant immunity in animals bearing growing primary tumors. To attempt to demonstrate altered immunity as a result of removal of the primary lesion by either cryosurgery or excision, and to study the response of metastatic deposits to this altered immunity.

Methods Employed:

Four different tumor-host systems were studied: A methylcholanthrene-induced sarcoma in CDF_1 mice, a mammary tumor virus induced mammary adenocarcinoma in both neonatally tolerant and virus free C3H mice, and two different SV40 induced tumors in hamsters. Tumor inocula were placed in the flank as previously described and when the tumors reached an appropriate size animals were treated by either cryosurgical destruction of tumor in situ or by excision. Some animals were left with their growing tumors. Several weeks later, normal control animals, the tumor cured animals, and the animals with either recurrence at the primary site or persisting untreated primary tumors were challenged with the same quantitative single cell suspension of tumor. The time of appearance of palpable tumor at the challenge site was noted, and the rate of growth of the challenge tumor was measured with vernier calipers as was the rate of growth of the primary or recurrent tumor when present. Some of the animals were selected for autopsy to determine the presence or absence of metastases after a sufficient period of time had passed to indicate whether the challenge inocula was going to grow or not.

Major Findings:

In all four of the tumor-host systems concomitant immunity was reproducibly demonstrable and seemed to be a general phenomenon. The degree of this immunity was intermediate between that demonstrable following cryosurgical destruction of the tumor in situ and that occurring after surgical excision. Rates of growth of primary tumor or recurrent tumors were unchanged following the challenge dose whether or not growth occurred from the challenge injection. In those animals autopsied, it was not uncommon to find that while the challenge inoculum was rejected, the primary tumor continued to grow and metastases developed and grew.

Significance to Bio-medical Research and Program of the Institute:

These data offer considerable support for the concept that metastases develop as a consequence of differential immunogenicity between the primary tumor and its metastases. The dosage of tumor cells employed in the challenges which demonstrated concomitant immunity was significantly greater than that dose assumed to have been released in the circulation to cause resultant formation of metastatic deposits. If the cells shed into the circulation are immunologically identical with the primary tumor and with the challenges rejected by concomitant immunity, it is difficult to envision that they would

successfully take and develop as a metastatic deposit in an animal immune to this tumor. Hence, the conclusion is inescapable that an animal rejecting a sizeable challenge in the presence of a growing tumor is unable to also reject the cells shed from the primary tumor to eventually form a metastasis because they are immunologically different from cells of the primary tumor. The consequences of this for eventual clinical application of immunotherapy are great in that most immunotherapy will be directed against metastatic disease. Hence, the differential immunogenicity of metastatic tumor may have to be seriously taken into account in developing modalities for immunotherapy.

Proposed Course of Project:

It is of some significance to ascertain whether or not the use of a single cell suspension is appropriate for challenge to demonstrate concomitant immunity. Current data suggests that metastatic deposits arise from clumps of shed tumor cells and not from single cells exfoliating into the blood stream. Consequently, this study will be repeated using clumped cell material rather than single cell suspensions as the challenge dose for testing concomitant immunity.

Project Description:

I. Immunity of the autogenous host to viable tumor cells shed from pulmonary metastases.

Objectives:

The phenomenon of preferential metastasis as vividly demonstrated by Kinsey was used to explore the autogenous biologic potential of viable tumor cells shed from pulmonary metastases. This study has direct bearing on the question, do metastases metastasize? and also has major implications for the immunity of the host to tumor cells circulating in the blood stream.

Methods Employed:

First, preferential metastasis was demonstrated in the fifth generation of a methylcholanthrene-induced sarcoma of C57 mice in syngeneic C57 black mice. Heterotopically transplanted organ fragments of newborn syngeneic C57 mouse lung, kidney and liver were aseptically placed subcutaneously. Forty-eight hours before transplantation of these organs the animals received a transplant of 10^6 tumor cells in the leg. Spontaneous metastases occurred only to pulmonary tissue, whether it be the animals own tissue or the transplant. Other organs and other transplants never developed tumor. In a second group of experiments, transplants were placed after amputation of a large leg primary, amputating having been performed at a stage where pulmonary metastases develop in the pulmonary transplant, and all the pulmonary metastases in the host were of approximately the same size. These data suggest that pulmonary metastases themselves are not capable of re-metastasizing to sites of

preferential metastasis. In a group of untreated animals, the left heart blood was removed and inoculated into syngeneic recipients. In 8 of 8 such bioassay recipients, tumor growth occurred, demonstrating the presence of viable circulating tumor cells shed from the pulmonary metastases in left heart blood.

Significance to Bio-medical Research and Program of the Institute:

It was concluded that within the context of this tumor-host system, which is a highly immunogenic tumor-host system, that metastases do not metastasize. The reasonable explanation for this finding was that tumor specific immunity, perhaps facilitated by removal of the primary lesion, prevented growth in the transplants which were previously sites of preferential metastases.

Publications:

Hammond, W.G. and Rolley, R.T.: Retained regional lymph nodes: Effect on metastases and recurrence after tumor removal. Cancer 25: 368-372, 1970.

Sugarbaker, E.V., Cohen, A.M., and Ketcham, A.S.: Facilitated metastatic distribution of the Walker 256 tumor in the Sprague-Dawley rats with hydrocortisone and/or cyclophosphamide. J. Surg. Oncol. 2: 277-289, 1970.

Articles In Press:

Sugarbaker, E.V., Ketcham, A.S., and Cohen, A.M.: Studies of dormant tumor cells. Cancer.

Sugarbaker, E.V., Cohen, A.M., and Ketcham, A.S.: Do metastases metastasize? Ann. Surg.

Serial No. NCI-3806

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Surgical Physiology and Metabolic Changes Associated with Extensive Cancer Surgery

Previous Serial Number: Same

Principal Investigator: Robert C. Hoye, M.D.

Other Investigators: A.S. Ketcham, M.D., S.H. Bennett, M.D., G.W. Geelhoed, M.D., and G.C. Riggle, B.S.

Cooperating Units: Department of Anesthesiology, Blood Bank, Department of Nursing, Radiopharmaceutical Service, and Biomedical Engineering and Instrumentation Branch, Clinical Center, NIH

Man Years: (July 1, 1970 through June 30, 1971)

Total:	2
Professional:	1
Other:	1

Project Description:

Part I: Albumin kinetics with trauma and hemorrhage in man and animals

Part II: Capillary permeability studies in man and animals

Part III: Studies on the etiology, pathogenesis, and treatment of the syndrome post-traumatic pulmonary insufficiency

Part IV: Feasibility studies on the use of auto-transfusion methods in major cancer surgery

Part V: Phasing out of laser energy studies on normal and malignant tissues

Project Description:

Part I: Albumin kinetics with trauma and hemorrhage in man and animals

Objectives:

Previous studies in this laboratory have indicated that after major cancer surgery a marked disproportionate plasma volume deficit occurs amounting to 24% of the patient's plasma volume immediately prior to surgery. In addition, a 33% deficit of the total circulating protein has been noted in patients immediately following major operative procedures. These studies are designed to quantitate the extent of albumin depletion in man and animal with operative trauma and to determine, if possible, the magnitude and direction of the shifts involved.

Methods Employed:

The plasma volume total exchangeable albumin, total circulating albumin, albumin distribution ratio, and fractional catabolic rate are being studied with conventional isotopic methods. When indicated patients are studied for 7-10 days at each measure interval and all losses measured at surgery and, in selected patients, whole body counting has also been employed. In measurement of the total circulating albumin in patients or animals, direct measurement of the plasma volume is determined at each measurement interval as well as determination of albumin by serum electrophoratic methods.

Major Findings:

In a series of nine patients undergoing regional node dissection, we have found that the total body albumin pool is decreased by 29% of the pre-operative measurement during the course of surgery. This amounted to 80 grams of albumin and only a small portion of this loss could be accounted for by external measurements. Whole body counting indicated that this albumin loss from the exchangeable body pool was sequestered in an uncirculating albumin pool which demonstrated a decrease of 27% over the time of the operative procedure. In control patients no changes in the albumin pool were noted. In view of the findings in these patients who underwent the regional node dissection it is anticipated that with operative procedures of two to three times the magnitude of a single regional node dissection it can be expected that between 160 and 240 grams of albumin will be sequestered.

In the 12 primates studied, it was found that intestinal manipulation caused a sequestration of albumin in the intestinal tract which amounted to 24% of the total circulating albumin pool present prior to the operation. In nine dogs as studied, it was observed that a simple non-shocking hemorrhage of 30% of the measured total blood volume resulted in a continued

additional loss of albumin from the intravascular compartment in the six hours following the hemorrhage which amounted to 33% of the total circulating albumin present in the circulation at the end of the hemorrhage period.

Other investigators had previously indicated that following hemorrhage in normal volunteers there was a rapid influx of albumin into the vascular compartment. Based on previous work in this laboratory this was not felt to be the situation in the postoperative patient and 13 patients were investigated. The total circulating albumin pool was measured before surgery. In the eight patients of the group who underwent major intra-abdominal resectional surgery the immediate postoperative total circulating albumin pool was 72% of the amount present in the preoperative period. This indicated net loss from the total circulating albumin pool of 28%. By six hours after surgery this deficit was 22% and at 12 hours after surgery this deficit was 19%. These studies indicated that following operative trauma and albumin sequestration there was not a rapid influx of albumin into the vascular compartment as had been suggested by other investigators following studies on normal volunteers but that instead there was a very slow influx which by 12 hours after the operative procedure had replenished only one-third of the deficit which had occurred during the surgical procedure.

Significance to Bio-medical Research and Program of the Institute:

These studies in animals and man indicate rather conclusively that with major surgery and trauma there is a sequestration of a significant portion of the total body albumin pool and that this amount of albumin remains sequestered for a considerable length of time. If normal homeostasis is to be maintained following the surgical procedure then large amounts of albumin infusions should be administered both during and after the operative procedure. If albumin is not administered the speed of plasma volume re-expansion after hemorrhage will be slowed and, in addition, there will be a lowering of the colloid osmotic pressure which may be responsible for the interstitial pulmonary edema occurring near the end of surgery or in the immediate postoperative period in some patients. Based on previous work it is felt that one of the major reasons for this loss of total circulating albumin is secondary to a 75% decrease in the amount of albumin being returned to the intravascular compartment via the thoracic duct lymph during the surgical procedure. In previous studies the thoracic duct has been cannulated and the flow rate and protein content of lymph being returned to the vascular system measured in patients undergoing surgery.

Metabolic changes following extensive cancer surgery have not previously been thoroughly investigated. To date the quantitative studies being carried out by this laboratory in patients undergoing extensive cancer surgery have indicated that a multiplicity of deficits may occur. With

the increased knowledge obtained in these studies the management of the postoperative patient has changed dramatically in recent years. The morbidity and mortality has decreased significantly with the institution of new treatment regimens.

Proposed Course of Project:

Future studies will continue to investigate these parameters in selected patients and animals while manipulating the type of replacement fluids given during the period of surgical stress.

Project Description:

Part II: Capillary permeability studies in man and animals

Objectives:

Patients who undergo extensive surgery in the treatment of cancer have been demonstrated to develop a disproportionate plasma volume deficit, a functional extracellular fluid volume loss, and a decrease in the total circulating albumin pool. To maintain adequate circulation these patients must be given large amounts of plasma, albumin, fluids, and electrolytes in the immediate postoperative period. There are reasons to believe that this phenomenon may be, at least in part, due to a generalized increase in capillary permeability.

Investigations in this area of study to date have been done by following the disappearance from the blood stream of radioactive labels. Such labels, at 60,000 molecular weight, may have been too large to identify significant changes in capillary permeability. A new method has been developed to measure capillary permeability over a wide molecular weight range and it is proposed to use this method in studying changes in capillary permeability with trauma and hemorrhage.

Methods Employed:

By using gel columns composed of sepharose, dextran can be fractionated into its various molecular weight components. It has previously been demonstrated using this method that the permeability of the capillary membrane to molecules from 10,000 to 200,000 can be determined with accuracy and reliability. The capillary permeability which occurs in response to hemorrhage, shock, and operative trauma will be followed in animals by following the disappearance from the blood stream of dextran molecules in molecular weight range of 10,000 to 100,000. Studies will be performed before and after the traumatic episode and the disappearance rates of the various molecular weight components of dextran administered will be compared. To facilitate interpretation of the results the renal excretion of the dextran molecules will be prevented by previous bilateral nephrectomy.

The permeability of various vascular beds will be studied in subsequent experiments as follows. At the time of nephrectomy the lymph channels draining the liver will be clamped, cut, and ligated at their point of entrance into the cisterna chyli. At the time of study, samples of the lymph from the thoracic duct and blood from the celiac axis and inferior mesenteric artery will be collected. After the fractionation of the dextran into its various molecular weight components the sieving characteristics of the intestinal circulation following a traumatic episode can be determined. In a similar manner, the sieving characteristics of the hepatic bed can be determined with prior ligation of the intestinal lymphatics to the cisterna chyli. The sieving characteristics of the pulmonary capillary bed will be studied by obtaining right thoracic duct lymph which drains the major portion of the pulmonary bed. Simultaneous with these studies measurements will be made of the plasma volume, extracellular fluid volume, total circulating albumin, serum colloid osmotic pressure, blood pH, and blood gases.

Depending upon the changes observed in these studies, attempts will be made to study the response of the general capillary bed to drugs and various fluid regimens administered at the time of the surgical trauma.

The experimental animals used in these studies will be those found to be most suitable for analyzing permeability characteristics of the capillary bed under investigation. For the intestinal and hepatic capillary beds it is felt the primate will be most suitable and for the study of the pulmonary capillary bed the larger calf or goat is anticipated to be a more suitable experimental animal.

Major Findings:

Using this method it has been demonstrated that after a period of hemorrhagic shock in splenectomized dogs that a slight increase in capillary permeability for molecular sizes below 40,000 occurred during and after the hemorrhagic shock phase. This occurred despite maintenance of a normal arterial tension.

The method has also demonstrated that after localized radiation injury there is a marked increase in capillary permeability for the fraction from 10,000 to 30,000 molecular weight. With the passage of time during the first two weeks after the radiation injury the amount of capillary permeability occurring increased.

Significance to Bio-medical Research and Program of the Institute:

The immediate significance of this work is improvement of the pre- and postoperative management of patients undergoing major cancer surgery.

Proposed Course of Project:

The major portion of this year has been used in a standardization of gel columns by using dextran fractions of normal molecular weight. In addition automatic methods for the determination of dextran have been developed on the autoanalyzer and are currently being programmed into a computer. It is anticipated that in the coming year significant results will be obtained to answer a few of the many questions concerning homeostasis which arised during the course of performing extensive cancer operations.

Project Description:

Part III: Studies on the etiology, pathogenesis, and treatment of the syndrome post-traumatic pulmonary insufficiency

Objectives:

In recent years some patients after major surgery have died in the immediate postoperative period of pulmonary insufficiency. This consisted primarily of patchy areas of interstitial pulmonary edema with resultant oxygen diffusion block, and the patient's death being due to anoxia. Multiple blood transfusions, microthrombi, fluid overload, and fat embolism have all been implicated as possible etiological factors.

The most significant pathologic process in the syndrome is one of the interstitial pulmonary edema, and capillary congestion. It is believed that the major factors involved in producing this process are increase in the capillary permeability, a decrease in the colloid osmotic pressure, and micro emboli in the pulmonary capillary. It is planned to investigate these parameters in both man and experimental animals during and following the course of surgical procedures.

Methods Employed:

Capillary permeability studies will be carried out with the gel diffusion dextran fractionation technique previously described and, in addition, serum colloid osmotic pressure will be measured on a special membrane colloid osmometer. Pulmonary artery pressures will be monitored as well and in patients arterial venous oxygen differences as well as alveolar-arterial oxygen differences will be monitored. With these methods clinically undetectable abnormalities in pulmonary profusion will be demonstrable. Micro emboli in the circulating blood or in blood to be transfused to patients during the surgical procedure will be investigated using an electromagnetic particle counter which counts in sizes abnormal particles in the blood. In addition, the screen filtration pressure apparatus will be employed to also investigate micro emboli in the circulating blood or in blood transfused.

In an experimental animal (dog, baboon) isolated perfusion studies, involving one lobe of the lung are being performed to demonstrate the damaging effects on the lung of such substances as aggregated platelets and white blood cells in stored blood, toxins present in blood obtained from septic animals, and blood with lowered colloid osmotic pressure.

In larger animals (calf, goat) the right thoracic duct is being cannulated in lymph collected. This lymph drain is primarily from the pulmonary bed and with circulating dextran of various molecular weights in the vascular system changes in the pulmonary capillary to lymph pore size will be demonstrable by passing the lymph through the previously described gel columns. Changes in capillary pore size will thus be demonstrable in response to the various insults delivered to the pulmonary vascular tree.

Major Findings:

In one of several patients studied thus far it has been observed following a radical hysterectomy during which the patient received eight units of blood, each of which was greater than 10 days old, that 12 hours following the surgical procedure arterial blood gas studies demonstrated a perfusion block in the pulmonary bed. This perfusion block necessitated an administration of oxygen by mask and did not clear up until 72 hours later. This abnormality in perfision which was present was not detectable clinically and lung scans and pulmonary angiograms demonstrated no evidence of pulmonary emboli. It is postulated that the defect exhibited by this patient was secondary to capillary plugging with aggregated platelets and white blood cells contained in the 10-day old transfused blood. It is believed that such abnormalities occur frequently although not clinically recognized. By routinely monitoring such patients for these abnormalities the perfusion deficit will be recognized earlier and treatment instituted immediately should prevent the development of progressive and irreversible pulmonary insufficiencies.

In the experimental animal isolated perfused lung system has been found to be very susceptible to minor changes in volume and perfusion pressures. By artificially aggregating platelets in the circulating perfused blood the pathological process of post traumatic pulmonary insufficiencies or congestive atelectasis has been reproduced. When blood from a septic animal was perfused through this system without changes in pressure or volume interstitial pulmonary edema occurred with some evidence of capillary obstruction. This finding suggests that the sepsis elsewhere in the body makes the lung respond in an abnormal fashion.

Significance to Bio-medical Research and Program of the Institute:

This syndrome of post traumatic pulmonary insufficiencies or congestive atelectasis has been recognized in recent years to be one of the main causes of death in patients following extensive surgery or trauma when a large number of blood transfusions have been administered. Similarly

pulmonary failure has been the primary cause of death in patients developing sepsis. By defining some of the factors occurring in this process it is anticipated that the detection of abnormalities which precede clinical manifestations of difficulty will lead to the institution of corrective measures before the process is in an irreversible state.

Proposed Course of Project:

Our primary effort in the coming year will be devoted to this projcct as it is this area of investigation which we feel will benefit the greatest number of seriously ill patients.

Project Description:

Part IV: Feasibility studies on the use of auto-transfusion methods in major cancer surgery

Objectives:

Auto-transfusion has been used with success over the past 80 years largely in emergency situations to restore blood volume following acute blood loss when no donor blood has been available. It has also been used successfully in a limited number of elective surgical cases but has not been used in oncologic surgery. This surgical area appears to be particularly applicable to auto-transfusion because of the significant blood loss encountered.

The purpose of this study is threefold: (1) to determine whether auto-transfusion is a safe and effective means of blood replacement in a patient undergoing extensive cancer surgery; (2) to examine specific effects of auto-transfusion on blood cellular elements, coagulation factors, circulating lipids and lipoproteins and (3) to develop a method of anti-coagulating blood suctioned from the operative wound without employing systemic anticoagulation.

Major Findings:

To date all studies have been done in animals in preparation for clinical trials in cancer patients undergoing major surgery.

To determine the effects of auto-transfusion on cellular elements and coagulation factors 15 dogs were divided into two groups and auto-transfused. Group I dogs were auto-transfused without entering the abdomen using the femoral vessels to show only the effects of the suction apparatus and pump. Group II dogs had midline abdominal incisions and were auto-transfused after suctioning blood from the peritoneal cavity.

The first group of dogs demonstrated a modest rise in free plasma hemoglobin but no other adverse changes in blood elements. The second group of dogs with open peritoneal cavities showed a greater rise in free plasma hemoglobin plus a transient decrease in platelets and an increase in fibrinogen. While statistically significant these changes did not appear to have clinical import.

Circulating lipoprotein and serum lipids were found to be unaltered by auto-transfusion in dogs.

A suction device has been fabricated by the Bio-engineering Division which will anticoagulate blood at the suction tip using the Venturi tube principle. This device has been tested in vivo and shown to be capable of delivering a consistent volume of Heparin solution per unit volume of blood aspirated regardless of whether blood alone or a blood air mixture is suctioned. Reversal of Heparin effects in the animal has been accomplished by "in-line" Protamine infusion through a double lumen intravenous catheter tip. Clotting studies indicate that this system is capable of maintaining complete anticoagulation in the extra corporeal circuit and normal clotting times in the animal.

Significance to Bio-medical Research and Program of the Institute:

Many deaths occur each year from post-transfusion hepatitis and other complications of donor blood transfusions. Patients undergoing exenterative surgery utilize large volumes of donor blood to replace that blood lost at surgery. The risk of hepatitis and fatal transfusion reactions is compounded by the number of units of blood used. If a method can be developed where by the blood lost from the patient at the time of surgery can be suctioned up and returned to the patient these deaths will be prevented by eliminating or decreasing the use of donor blood for transfusion.

Proposed Course of Project:

With the refinement of the anticoagulation system and the acquisition of disposable filtering devices already used clinically for auto-transfusion we anticipate clinical trials in the near future.

Project Description:

Part V: Phasing out of laser energy studies on normal and tissues

Proposed Course of Project:

Due to the lack of reproducible energy outputs and the failure of being able to develop reliable clinical interpretation of the experimental

results, the program was phased out. The laser equipment has been assigned to the Bio-medical Engineering and Instrumentation Branch for future developmental work.

Publications:

Riggle, G.C., Hoye, R.C., Farmer, J., Ketcham, A.S.: Q-Switched Neodymium laser effects on tissue. Record of 10th Symposium on Electron, Ion, and Laser Beam Technology (L. Morton, ed.) San Francisco Press, Inc., 1969, pp. 487-492.

Ketcham, A.S., Hoye, R.C., and Riggle, G.C.: The laser in cancer research. Ann. N.Y. Acad. Sci.168: 634-641, 1970.

Hoye, R.C., Paulson, D.F., and Ketcham, A.S.: Total circulating albumin deficits occurring with extensive surgery. Surg. Gynec. Obstet. 131: 943-952, 1970.

Ketcham, A.S., Hoye, R.C., and Riggle, G.C.: The laser: Its role in cancer. In Ariel, I.M. (ed.): Progress in Clinical Cancer. New York, Grune-Stratton, 1970, pp. 109-117.

Riggle, G.C., Hoye, R.C., and Ketcham, A.S.: Laser effects on normal and tumor tissue. In M.L. Wolbarsht (ed.): Laser Applications in Medicine and Biology. New York, N.Y., Plenum Publishing Corp., 1970, pp. 35-65.

ARTICLES IN PRESS

Hoye, R.C., Voightlander, J., Birke, G., Plantin, L.O.: Changes in the total circulating albumin, plasma volume and extracellular fluid volume with hemorrhage and the response to cortisone. Acta Chir, Scand., in press.

Serial No. NCI-3813

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Surgical Endocrinology

Previous Serial Number: Same

Principal Investigator: J.R. Marshall, M.D.

Other Investigators: J.A. Wider, M.D., G.T. Ross, M.D., C.B. Cargille, M.D., R. Henkin, M.D., N. Sato, M.D., and A. Simopolus, M.D.

Cooperating Units: Endocrinology Branch, NCI; Pathologic Anatomy Branch, NCI; Reproductive Biology Branch, NICHD; Endocrinology Branch, NHLI

Man Years: (July 1, 1970 through June 30, 1971)

Total:	4
Professional:	1
Other:	3

Project Description:

Part I. Pharmacology of Gonadotropins.

Part II. Studies of Pregnancy and Trophoblastic Disease.

Part III. Gonadotropin Control.

Part IV. Evaluation of the Anovulatory Female.

Project Description:

Part I. Pharmacology of Gonadotropins.

Objectives:

To clarify the significant variables in ovulation induction using human menopausal gonadotropins; and to develop a safe, effective and applicable treatment technique.

Methods Employed:

Using a standard threshold dose of human menopausal gonadotropin by means of individual patient dose response curves, variables controlling response to both human menopausal gonadotropin and human chorionic gonadotropin are studied in anovulatory women.

Major Findings:

Estrogen response, ovulatory response rate, and ovarian enlargement rate are positively dose related. Oligo-ovulatory patients have lower thresholds to human menopausal gonadotropin (HMG) than do anovulatory patients. Effectiveness of varying dosage schedules is determined by patient threshold. Patients requiring less than 1,000 i.u. follicle stimulating hormone (FSH) per cycle respond to a single injection of the entire dose; patients requiring larger gonadotropin dose need divided doses. The FSH and luteinizing hormone (LH) content of the material are both of importance in determining ovulatory rate; however, FSH content is more critical. Low LH materials are less effective in inducing ovulation than are materials with greater amounts of LH. Pretreatment urinary gonadotropin levels are not determinants of responsiveness unless very high levels are found. Urinary steroid studies likewise are not helpful in selecting dose. Plasma LH is of some help in predicting gonadotropin dose. Plasma FSH levels are of little use unless markedly elevated.

Simultaneous administration of ethinyl estradiol with HMG to patients with intact pituitaries results in a decreased plasma FSH, no consistent change in plasma LH and a decreased ovulatory rate when compared to administration of HMG without estrogen. However, these differences are not seen in patients following hypophysectomy and in whom estrogen appears to enhance the response to HMG.

Ovulatory rate and corpus luteum function as measured by plasma progesterone levels are both dependent upon the human chorionic gonadotropin (HCG) dose used to induce ovulation. Plasma levels of HCG following injection are dependent upon the HCG dose. Peak levels occur within 12 hours of injection and return to control values by five to seven days. A single injection of 5-10,000 i.u. of HCG seems to be optimal for induction of ovulation.

Based on these considerations a technique of dose selection has been developed which is widely applicable, safe and effective. It uses gradually increasing doses of HMG followed by a single injection of HCG to delineate the HMG ovulatory threshold and then to induce ovulation for pregnancy.

Significance to Bio-medical Research and Program of the Institute:

Human menopausal gonadotropin promises to be a useful agent in the study of and treatment of anovulatory women. The present problems associated with this drug are such as to preclude its widespread use. It is our feeling that these unwanted side effects are largely dose related and could be

eliminated by more appropriate dose selection. In this regard, the information derived from this study will provide a basis for valid selection of dosage.

Proposed Course of Project:

Project is continuing with attention to additional variables and to more basic physiology of response.

Project Description:

Part II. Studies of Pregnancy and Trophoblastic Disease.

Objectives:

To gain a better understanding of the natural history of benign and malignant trophoblast.

Methods Employed:

Plasma HCG radio-immunoassay, competitive protein binding assay of plasma, progesterone and 17 hydroxyprogesterone.

Major Findings:

Plasma HCG concentrations are elevated over control values by conception day 11 and thereafter rise exponentially until at least day 21. Doubling time is 1.7 days. Geometric consideration of the developing ova suggests that the trophoblast mass increases as does the diameter to the second power. When the maximum diameter of the trophoblast is increased to the second power and its rate of increase (early human ova collected by Hertig and Rock) compared to the rate of increase of HCG concentration, no significant difference is seen. This suggests that plasma HCG concentrations mirror trophoblast mass.

Rates of change in plasma HCG levels in patients with trophoblastic disease in regression, remission and in relapse on and off chemotherapy have been compared statistically. On chemotherapy plasma HCG falls exponentially with a maximal half time of about 3.5 days. This compares with a half time of 23 hours following removal of the placenta and normal delivery. There does not appear to be any correlation between rate of decline and prognosis. Rate of increase either on or off chemotherapy appears to fall into two categories. One with a doubling time of 2.5 days and the other with a doubling time of 15 days.

Significance to Bio-medical Research and Program of the Institute:

Chemotherapy of gestational trophoblast neoplasia has been based upon the assumption that gonadotropin excretion adequately reflects residual tumor. The present study substantiates this assumption. Moreover, utilizing this

assumption the study of rates of change of HCG levels in patients with trophoblastic disease now can provide information about the rate of change of tumor cells and open the door to studies of the cell kinetics of human neoplasia.

Proposed Course of Project:

Further analysis of data with examination of data in terms of cell kinetics.

Project Description:

Part III. Gonadotropin Control.

Objectives:

To understand the mechanisms of gonadotropin control of the female reproductive process.

Methods Employed:

Patients under study and treatment for infertility were studied with various surgical and endocrinological techniques and results correlated with clinical parameters.

Major Findings:

In patients responding with ovulation Clomiphene citrate induces a pattern of gonadotropin response very similar to that noted in the normal ovulatory cycle. FSH reaches a peak on day three or four and thereafter declines to a preovulatory nadir. LH reaches a peak on day six or seven and then declines. Both FSH and LH rise and then fall precipitously at mid-cycle, and then persist at low levels during the luteal phase.

In patients not ovulating to Clomiphene citrate several patterns of response have been seen. An early "Clomiphene follicular" peak in both FSH and LH can occur without a mid-cycle peak. In these patients administration of HCG on day 12-15 frequently results in ovulation. An early peak in FSH can occur without any change in LH. An increase in LH can occur without change in FSH and this rise can be followed by a sustained and elevated, but spiking LH response. In some individuals no change in plasma gonadotropins is seen. Delineation of these patterns of response to Clomiphene enables patients to be classified according to these patterns and separates the functional centers controlling ovulation.

Oxytocin has been reported to be effective as a releaser of gonadotropins. The effect of a prolonged intravenous oxytocin infusion of plasma FSH and LH has been studied. No release of gonadotropins has been seen.

Proposed Course of Project:

Additional studies using Clomiphene, oxytocin, chlorpromazine, and other hypothalamic stimulators and blockers are either underway or planned.

Project Description:

Part IV. Evaluation of the Anovulatory Female.

Objectives:

To expand and improve the diagnosis and classification of anovulation in the female.

Methods Employed:

Diagnostic culdoscopy, operative culdoscopy, progesterone uterine stimulation, Clomiphene-hypothalamic-pituitary stimulation and classification according to dose-response relationships and gonadotropin ovarian stimulation and classification with each being correlated with clinical findings.

Major Findings:

The presence or absence of the normal ability to smell and taste correlates well with the presence or absence of oocytes in women with primary amenorrhea. A classification of functional hypothalamic disorders is being made on the basis of the clinical response to gonadotropins and delineation of unusual patients with oocytes resistant to gonadotropins has been made.

Proposed Course of Project:

Project will continue along same general lines.

Publications:

Marshall, J.R.: Ovulation induction. Obstet. Gynec. 35: 963-970, 1970.

Wider, J.A. and Marshall, J.R.: Hysterotomy and insertion of an intrauterine device for endometrial sclerosis: Importance of long-term followup. Fert. Steril. 21: 240-243, 1970.

Marshall, J.R. and Wider, J.A.: Results of human menopausal gonadotropins (HMG) therapy for anovulatory infertility using a nonvariable treatment schedule: Comparison with previous reports. Fert. Steril. 22: 19-25, 1971.

Serial No. NCI-3816

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cryobiology

Previous Serial Number: Same

Principal Investigators: W.G. Hammond, M.D., and A.S. Ketcham, M.D.

Other Investigators: H.B. Neel, III, M.D., and Mr. Grant Riggle

Cooperating Units: A.S. Rabson, M.D., Pathologic Anatomy Branch, NCI

Man Years: (July 1, 1970 through June 30, 1971)

Total:	1½
Professional:	½
Other:	1

Project Description:

Part I. Effects of Cryonecrosis on Development of Tumor Specific Immunity.

Part II. Immunity Following Cryonecrosis of Mammary Tumor Virus-Induced Tumor in Tolerant and Nontolerant Animals.

Part III. Cryodestruction of a Functional Endocrine Tumor.

Part IV. Cryosurgery of Liver.

Part V. Cryosurgical Studies in a Functional Nonendocrine Tumor.

Part VI. Correlation Between Various Freezing Conditions, Temperature Measurements in the Frozen Tissue, and Biologic Effects of the Freeze.

Project Description:

Part I. Effects of Cryonecrosis on development of tumor specific immunity.

Objectives:

To ascertain whether cryosurgical destruction of tumor results in the development of an immunologic response to tumor specific antigens which is different from that response resulting from excision of tumor in a similar tumor-host system.

Methods Employed:

Three new tumors were studied in this portion of the project during the past year. The first was a previously unstudied carcinogen-induced sarcoma in inbred mice. The other two were a prostatic-like SV-40 virus-induced tumor and a nonprostatic tumor of a similar origin, both in random bred hamsters. Standardized growth was achieved with single cell suspension inocula. Methods of positioning tumor implant and freezing the tumor nodule were those previously developed. In some instances the tumor was excised surgically instead of being destroyed by cryosurgery. At standard times following removal of tumor by cryosurgical destruction or by excision, all animals without residual tumor as well as appropriate controls were then challenged with standard single cell suspension inocula of the same tumor. Time of development of palpable tumors was recorded as the animals were observed frequently for tumor growth following challenge. Tumor sizes were also measured with a vernier caliper.

Major Findings:

In the mouse carcinogen-induced tumor system, excision and cryosurgical tumor destruction both resulted in demonstrable immunity to subsequent challenge. However, those animals cured by cryosurgery were significantly more immune than were the animals cured by excision, as assessed by any of the standard methods for evaluating immunity in these systems; i.e., latency period of tumor development following challenge, rate of tumor growth as measured volumetrically and, most significantly, incidence of final tumor take at the conclusion of the experiment.

With respect to the SV-40 virus-induced tumors, as expected there was cross immunity between the prostatic-like and the nonprostatic tumor. Evaluation of the data with respect to differential immunity between cure by excision and cure by cryosurgery is still incomplete. However, there is no striking differential immunity and it is expected that significant differential immunity will not be demonstrable in this particular tumor system. The reasons for this are not immediately apparent since there was demonstrable differential immunity as noted in this report last year in other virus-induced tumors. It is believed that the fact that these studies were done in non-inbred animals may have a bearing on the absence of differential immunity, since varying amounts of minor histocompatibility differences may cancel out any differential immunity due to the mode of cure of the tumor.

Attempts to demonstrate immunologic effects on subsequent growth of occult metastases or small foci of residual tumor have thus far been unsuccessful. This is largely a consequence of the great difficulty of achieving a standard system of occult metastases without unduly sacrificing great numbers of animals.

Preliminary data from studies in which temporary ischemia of tumor (produced by rubber band compression of the pedicle leading to subsequent regression and disappearance of tumor) as a method of tumor destruction suggests that this produces a differential immunity similar to that produced by cryosurgical destruction in situ and that destruction by intense cold per se does not alter immunity to subsequent challenge, but that duration of exposure to TSTA(s) is an important factor.

Significance to Bio-medical Research and Program of the Institute:

There are both data in experimental systems and analogous clinical observations which suggest that in situ kill of sizable amounts of tumor, without removal of the dead tumor, will result in an immunologic response to tumor specific antigens as great or greater than that occurring as a result of temporary tumor growth followed by surgical excision. These experiments were designed to test this hypothesis which now is receiving wide acclaim in the clinical surgical literature but has been based thus far on insufficient experimental evidence. These data suggest that this concept may be valid since it is demonstrable in several tumor-host systems. It also appears valid in the respect of different ways of producing in situ cure without removal of the dead tumor, i.e., cryosurgery and temporary complete ischemia. If further substantiated by experiments in progress, these results will have readily apparent clinical significance for efforts to control tumor at present unresectable by conventional surgical means.

Proposed Course of Project:

The studies concerned with ischemic tumor kill will be more fully evaluated and supplementary experiments carried out as necessary.

Project Description:

Part II. Immunity following cryonecrosis of mammary tumor virus-induced tumor in tolerant and nontolerant animals.

Objectives:

This project was designed to assess the differential effect of cryosurgical destruction of the same virus-induced tumor in hosts which are neonatally tolerant to the virus and in those which are nontolerant and virus-free.

Methods Employed:

Early transplant generations of a mammary tumor virus-induced C_3H/HeN mammary adenocarcinoma were used. Two groups of mice were used as hosts. One group received the virus from their mother by vertical transmission while the other group was a histocompatible strain of mice rendered free of the virus by foster nursing on virus-free mothers immediately after birth. Tumor implants were made in the flank and cryosurgically destroyed or excised when they were 1 cm. in diameter. Groups of both tolerant and virus-free non-tolerant animals were treated by both modalities. Two weeks following either removal or destruction of tumor in both strains of mice, the mice were challenged with quantitative single cell suspensions of the same tumor. Appropriate controls were similarly challenged. Animals were observed carefully and the time of first detectable tumor appearance at the site of challenge of injection was noted. Rate of growth of tumors was measured with vernier calipers.

Major Findings:

Tumor immunity was readily demonstrable in animals cured of their tumor by cryosurgery whether they were tolerant or nontolerant of the virus. However, in the mammary tumor virus tolerant line of animals, following cure of tumor by excision, there was only slight immunity and in the mammary tumor virus-free line, there was some enhancement of tumor growth following cure by surgical excision. Whether this enhanced growth is due to persistent viremia or to an increase in the enhancing component of the immunologic response in these tolerant mice is at present unclear. However, in both tolerant and virus-free mice, there was significant differential immunity between the two methods of tumor cure, the immunity in both instances being stronger in those animals cured by cryosurgery.

Significance to Bio-medical Research and Program of the Institute:

These results are contrary to the original hypothesis developed at the beginning of the experiments. It was expected that nontolerant mice would demonstrate comparable immunity following cryosurgical necrosis or excision of tumor, whereas tolerant mice would demonstrate little or no immunity after excision but would have definite immunity after cryosurgical destruction. However, immunity seems greater after cryosurgical tumor destruction in both tolerant and virus-free mice. This enhances the prospect of successfully applying cryosurgery as a modality for treatment of putatively virus-induced human breast neoplasms.

Proposed Course of Project:

Publication of data.

Project Description:

Part III. Cryodestruction of a functional endocrine tumor.

Objectives:

To evaluate the efficacy of cryosurgery for a functional endocrine tumor with a readily assessable physiologic endpoint.

Methods Employed:

The tumor-host system used in these studies was a transplantable hamster islet cell adenocarcinoma in the 90 to 100th transplant generation. The tumor is freely transplantable among Syrian hamsters and is functional with significant hyperinsulinism when the tumor reaches 607 mm. in diameter. Tumor was injected and treated cryosurgically by the methods described above. By the time tumor became big enough to treat, the hamsters were significantly hypoglycemic and many seizures were occurring. Following cryosurgical destruction of tumor, a prompt rise in glucose levels occurred, and sequential blood sugar determinations revealed a return to normal levels. Using a double freeze technique and a larger probe than previously, a significant number of cures were obtained. In these animals the blood sugar remained normal indefinitely. Controls of the same strain revealed no significant changes in blood glucose levels following cryosurgical treatment of a different and nonfunctional tumor. Persistent hypoglycemia following apparently successful local eradication of tumor suggested occult metastases which were confirmed at autopsy or by the subsequent clinical course. Studies of blood insulin levels are still preliminary but the insulin level appears to correlate satisfactorily with the blood sugar levels.

Significance to Bio-medical Research and Program of the Institute:

The demonstration that abnormal biochemical findings may return to normal promptly after cryosurgical treatment of the neoplasm is of potential significance concerning clinical applicability of cryosurgery. There are many functional neoplasms which produce distressing side effects as a result of the endocrine abnormality, and patients may even die from severe endocrine abnormalities long before progressive spread of tumor would result in their demise. Since cryosurgical interruption of the endocrine abnormality may be effective even without complete tumor control, this may offer considerable palliation to such patients through the relatively safe modality of cryosurgery.

Proposed Course of Project:

Publication of data.

Project Description:

Part IV. Cryosurgery of Liver.

Objectives:

To assess the pathophysiologic consequences of producing cryosurgical destruction of a significant proportion of liver, and to evaluate the effect of transient hepatic ischemia at the time of cryosurgery on the size and development of the subsequent cryolesion.

Methods Employed:

Initial studies were done in rats. A 2 to 2½ cm. iceball lesion was produced cryosurgically. The effects of multiple freezes and the effects of ischemia by occlusion of the porta hepatis were both studied. Effects of lesions at more than one site in the liver were also studied, In young Rhesus monkeys, 2-1/2 cm. cryolesions were similarly produced in either lobe of the liver. Additionally, both repeated freezings at one site and periods of transient ischemia were studied. Multiple serial determinations of hematocrit, white count, serum alkaline phosphatase, bilirubin, SGOT, and SGPT were performed. At a certain time after the freeze, animals were sacrificed, the area of freeze inspected grossly and photographed, and specimens obtained for histologic examination.

Major Findings:

In neither rats nor monkeys was there any untoward systemic complication of the liver freeze. There was no bleeding, no jaundice, and no progression to massive liver necrosis. The degree of elevation of serum transaminases was well correlated with the extent of cryonecrosis. Repetitive freezing at a single site produced greater area of eventual cryonecrosis than did a single freeze. Temporary ischemia with a single freeze produced greater necrosis than did a single freeze alone. In both instances this increased cryolesion was accompanied by greater elevations of serum transaminases. Sixty-five days after producing a cryolesion in monkey liver, there was a very small scar which grossly is virtually undetectable. There were no late demonstrable abnormalities of liver function and immediately prior to sacrifice all monkeys appeared quite normal.

Significance to Bio-medical Research and Program of the Institute:

These studies demonstrate that significant amounts of the primate liver may undergo aseptic cryogenic necrosis without the development of hemorrhagic or biliary tract complications. Resultant scar formation does not significantly involve adjacent spread to liver or adjacent viscera. These data provide a hopeful outlook for the potential of the use of cryosurgery in the palliative management of hepatic metastases in patients.

Proposed Course of Project:

Publication of data.

Project Description:

Part V. Cryosurgical studies in a functional nonendocrine tumor.

Objectives:

These studies were undertaken to assess some of the parameters involved in the cryosurgery of an experimental prostatic cancer. As this tumor produces elevated serum acid phosphatase levels, attempts were made to assess the relationship between tumor destruction and change in serum acid phosphatase levels.

Methods Employed:

This transplantable hamster prostatic carcinoma, induced by *in vitro* SV-40 virus transformation of fetal hamster prostatic tissue, was injected in single cell suspensions in the flank and treated cryosurgically as previously described. The tumor metastasizes diffusely after reaching approximately 1 to 1½ cm. in diameter locally. Serum acid phosphatase levels were measured and the tartrate inhibitable fraction determined. (This component will be referred to subsequently as prostatic acid phosphatase.)

Major Findings:

The tumor is curable by cryosurgery only by using a large probe to increase the spread of freeze and delay as the time of thawing. An interesting finding was that elevated serum acid phosphatase, both total and prostatic fraction, is demonstrable prior to the time that the tumor becomes palpable. Following cryosurgical treatment, if there is a permanent cure, both serum acid phosphatases fall to normal levels and remain there. If the serum acid phosphatase values do not return to normal or transiently return to normal and again rise, either occult metastases are present and later develop clinically, or there will be a recurrence at the primary site. This tumor system has also been studied in attempts to demonstrate differential immunity as noted above. It is of interest that while immune animals do not develop palpable tumor from the subsequent challenge, a definite although transient rise in serum acid phosphatase values occurs following injection of the challenge dose of tumor.

Significance to Bio-medical Research and Program of the Institute:

This model promises to be useful for a number of studies because of the remarkably constant correlation between elevated serum acid phosphatase values and the presence of viable tumor in the animal although tumor is not demonstrable by other than histologic methods.

Proposed Course of Project:

Publication of data.

Project Description:

Part VI. Correlation between various freezing conditions, temperature measurements in the frozen tissue, and biologic effects of the freeze.

Objectives:

The objectives of this study were to evaluate the effects of altered physical conditions of freezing on the time course of temperature changes at various sites in the frozen area, and to correlate this with the biologic effects of cryothermal insults thus achieved.

Methods Employed:

These studies were done in several of the tumor systems described above, and in the normal rat liver. Two different probe tip temperatures were used, three different sizes of probe tips with varying tip to tissue contact areas were employed, and the effect of repetitive freezing was assessed. Needle thermacouples were inserted into the tissue to be frozen at varying distances from the cryoprobe, and continuous recordings made. Additionally, in many experiments the time taken to achieve a standard size iceball (as measured with calipers) during the process of freezing was noted with a stopwatch. In the tumor systems, cure rate was correlated with the various freezing conditions. In the normal rat liver system, extent of late cryolesion was correlated with various freezing conditions.

Major Findings:

Although in the vast majority of these studies the standard endpoint was the development of a standard size visually determined iceball, as temperatures were measured in the frozen tissue rather striking differences were obtained, depending upon the freezing conditions. As an example, using a medium size probe tip at a temperature of minus 60^{o}, only a small central fraction of the area frozen achieved a temperature significantly below 0^{o} Centrigrade. However, using a larger probe tip at a temperature of minus 180^{o} C. As would be expected, with the freezes carried to a deeper temperature there was greater strikingly prolonged thawing time. Additionally, when the more severe conditions of freezing were utilized (maximum probe tip tissue contact and deepest temperature) the time required to achieve the standard size iceball was significantly shortened. This time was also shortened with a second and a third freeze of the same area following thawing of the previous freezes. In the liver system, the second and third freezes under constant freezing conditions achieved lower temperatures and prolonged thawing times.

Significance to Bio-medical Research and Program of the Institute:

These data demonstrate that visual monitoring of cryosurgery by only noting iceball diameter is a grossly inadequate method for ascertaining the biologic effectiveness of the cryothermal insult. With comparable visual iceballs, the measured temperatures are strikingly different. This difference is reflected in both the tumor cure rate and extent of cryolesion in normal liver. It is obviously apparent that initial clinical efforts to employ cryosurgery in the treatment of human cancer will require careful thermacouple temperature monitoring so that a reliably predictable cryothermal insult can be delivered to the tumor.

Publications:

Myers, R.S., Hammond, W.G., and Ketcham, A.S.: Cryosurgical necrosis of the head of the pancreas. Ann. Surg. 171: 413-418, 1970.

Neel, H.B., III, Neel, H.B., and Hammond, W.G.: A reappraisal of primary gallbladder carcinoma. J. Surg. Oncol. 2: 131-143, 1970.

Neel, H.B., III, Ketcham, A.S., and Hammond, W.G.: Comparison of tumor immunity after complete excision, cryonecrosis, and in the presence of persistent tumor. Surg. Forum 21: 120-122, 1970.

Neel, H.B., Ketcham, A.S., and Hammond, W.G.: Requisites for successful cryogenic surgery of cancer. Arch. Surg. 102: 45-48, 1971.

Articles in Press:

Neel, H.B., Ketcham, A.S., and Hammond, W.G.: Ischemia potentiating cryosurgery of primate liver. Ann. Surg., in press.

Serial No. NCI-3817

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Subcellular Mediation of Immune Responses to Tumor Antigens

Previous Serial Number: Same

Principal Investigator: Yosef H. Pilch, M.D.

Other Investigators: P.J. Deckers, M.D. and B.R. MacPherson, M.D.

Cooperating Units: None

Man Years: (July 1, 1970 through June 30, 1971)

Total:	3-1/2
Professional:	1
Other:	2-1/2

Project Description:

Part I. The Immunoreactivity of Heterologous RNA in Mediating Immune Responses to Tumor Isografts in Inbred Mice.

Part II. Mediation of Immune Responses to Tumor Specific Transplantation Antigens by "Immune" RNA as Demonstrated by Immune Cytolysis.

Part III. Demonstration that Heterologous RNA is Incorporated into Spleen Cells.

Part IV. Demonstration of Immunoreactivity of RNA in Mediating Immune Responses to Tumor Specific Transplantation Antigens In Vivo.

Part V. Direct Injection of "Immune" RNA Protecting the RNA from Degradation by Admixture with RNAase Inhibitors.

Objectives:

To isolate preparations rich in ribonucleic acid from cells and tissues which process antigens, such as lymph nodes and spleen of animals immunized with tumors and to study the immunoreactive properties of these RNA preparations in mediating immune responses to tumor antigens. To utilize these preparations for immunotherapeutic purposes in experimental animal models.

Methods Employed:

RNA is prepared by phenol extraction for lymph nodes and spleens or peritoneal macrophages of experimental animals following immunization in vivo or in vitro with tumor cells or following immunization of animals by temporary tumor growth. RNA is determined by the orcinol reaction, DNA by the diphenylamine reaction, and protein by the Lowery method. RNA is characterized by its sedimentation characteristics upon ultracentrifugation on sucrose density gradients. The tumor-host systems employed have been benzpyrene-induced sarcomas in inbred mice, methylcholanthrene-induced sarcomas in inbred mice, benzpyrene-induced sarcomas in inbred rats, and methylcholanthrene-induced sarcomas in inbred mice, benzpyrene-induced sarcomas in inbred rats, and methylcholanthrene-induced sarcomas in inbred guinea pigs. RNAase levels are determined by a modification of the method of Roth and Hurley. Cellular immune responses are evaluated by modifications of the methods of Granger and Weiser and by a modification of the immune cytolysis assay of McKhann.

Major Findings:

Part I. The immunoreactivity of heterologous RNA in mediating immune responses to tumor isografts in inbred mice.

Hartley guinea pigs are immunized with early transplant generations of a benzpyrene-induced sarcoma induced and carried in C_3HFB mice. Lymph nodes and spleens are removed and RNA extracted from these lymphoid organs. This RNA is then incubated with normal, non-immune C_3H spleen cells which are then injected intraperitoneally into normal, previously untreated C_3H mice who are then challenged with inocula of viable tumor cells from the same sarcoma used to immunize the Hartley guinea pigs. Statistically significant reductions in the incidence of tumor development has been observed in animals so treated. Immunization of Hartley guinea pigs with normal C_3H mouse tissues, i.e., lung, liver, spleen, heart and kidney and use of RNA from lymph nodes and spleens of pigs so immunized has failed to cause statistically significant reduction in the incidence of growth of tumor isografts. Treatment of the active RNA preparations with RNAase abrogates the response. Treatment of the RNA preparations with DNAase or with pronase does not remove activity.

Specificity for the immunizing tumor was shown in that animals treated with spleen cells incubated with RNA from the lymphoid organs of Hartley guinea pigs immunized to one benzpyrene-induced sarcoma resulted in statistically significant reduction in the incidence of growth of tumor isografts of that tumor but not isografts of another benzpyrene-induced mouse sarcoma. Immune RNA from guinea pigs immunized to the second murine sarcoma transferred immunity to that sarcoma but not to the first.

Under another set of experimental circumstances, enhancement or facilitation in the growth of tumor isografts can be accomplished. In these experiments, RNA-incubated spleen cells, as described above, are mixed with tumor cells in vitro and then the mixture injected subcutaneously into previously untreated normal C_3H mice. Facilitated growth of tumor isografts is then observed. When sera are obtained from such animals, the presence of humoral antibody in these sera can be demonstrated by incubating tumor cells with these sera, in vitro, and then injecting suitable inocula of these cells into susceptible hosts. Under these circumstances, enhanced growth of such tumor cell inocula is observed.

Part II. Mediation of immune responses to tumor specific transplantation antigens by "Immune" RNA as demonstrated by immune cytolysis.

These experiments have been carried out in an entirely syngeneic tumor-host system, i.e., the RNA donor, spleen cells, and tumor-host system are all from the same inbred strain of rodent. In this phase of the work, inbred strain 2 guinea pigs are hyperimmunized with a methylcholanthrene-induced liposarcoma by excising a growing tumor and then, over a two to three month period, giving repeated inocula of viable tumor cells subcutaneously and intraperitoneally. Spleens are then harvested and RNA extracted from the spleens. This RNA is then incubated with normal, non-immune strain 2 spleen cells and concentrated droplets of these RNA-incubated spleen cells are dropped on monolayers of the liposarcoma grown in tissue culture. Following incubation for 48 hours at 37°C acellular areas of plaques in the tumor cell monolayers are observed corresponding to the areas of the spleen cell droplets. Normal spleen cells do not produce plaques; spleen cells incubated with RNA from guinea pigs immunized with Freund's adjuvant only or never immunized do not cause zones of lysis in the liposarcoma monolayers. RNA treatment of the active RNA preparations again abrogates the response. Spleen cells incubated with RNA extracted from non-lymphoid organs of guinea pigs does not mediate the response. Spleen cells incubated with preparations rich in solubilized tumor specific transplantation antigens prepared from the liposarcoma either after the method of Oettegen or by the method of Holmes failed to produce zones of immune cytolysis in the monolayers. When spleen cells incubated with RNA extracted from animals immunized to the liposarcoma are dropped on monolayers of a methylcholanthrene-induced strain 2 guinea pig osteosarcoma whose tumor specific transplantation antigens do not crossreact with those of the liposarcoma, no significant

immune cytolysis is produced. This demonstrates the specificity of the reaction for the immunizing tumor. Spleen cells incubated with RNA extracted from animals immunized with either strain 2 guinea pig sarcoma failed to mediate immune cytolysis on monolayers of strain 2 embryonal lung epithelial cells.

Part III. Demonstration that heterologous RNA is incorporated into spleen cells.

Hartley guinea pigs were immunized with the benzpyrene-induced murine sarcoma and were then pulsed with tritiated uridine. RNA was extracted in the usual fashion and incubated in vitro with C_3H mouse spleen cells. Radioautography was performed and intracellular granules were noted demonstrating incorporation of the labelled guinea pig RNA into the mouse spleen cells. The addition of a large excess of cold uridine did not cause a diminution in the number of intracellular granules demonstrating that labelled RNA rather than uridine alone was incorporated.

Part IV. Demonstration of immunoreactivity of RNA in mediating immune responses to tumor specific transplantation antigens in vivo.

For these experiments, again, a completely syngeneic tumor-host system was employed in which the RNA donors, spleen cells, and tumor were all from the same inbred rodent strain. In this case, a benzpyrene-induced sarcoma in inbred Fisher 344 rats was employed. Fisher 344 rats were hyper-immunized to the tumor by excising growing tumor transplants and then giving weekly inocula of viable tumor cells subcutaneously and intra-peritoneally. Spleens were then excised and RNA extracted. This RNA was then incubated with normal, non-immune Fisher spleen cells and previously untreated Fisher rats were inoculated intravenously with these RNA incu-bated spleen cells and challenged subcutaneously with a counted tumor cell inoculum. In one experiment, although no significant decrease in tumor incidence was observed, the animals receiving RNA-incubated spleen cells evidenced a prolonged latency period, statistically significant diminution in tumor volumes and prolongation of survival. In another experiment, marked decrease in cumulative tumor incidence was produced. Again treat-ment of the immune RNA with RNAase abrogated the response.

Part V. Direct injection of "Immune" RNA protecting the RNA from degradation by admixture with RNAase inhibitors.

A detailed study has been performed in our laboratory on the inhibition of ribonuclease activity caused by polyanions. Dextran sulfate, sodium salt of varying molecular weights has been studied in particular. These studies were performed using purified bovine pancreatic ribonuclease and dextran sulfate of clinical grade with molecular weights ranging from 25,000 to 2,000,000. These studies demonstrated significant inhibition of ribonuclease

activity, which was not present at molecular weight 25,000 and was minimal at 47,000, was significant at 77,000, greater at 250,000 and reached a maximum at molecular weights 500,000 and 2,000,000. Guinea pigs were then immunized to the C_3H murine sarcoma, lymph nodes and spleens were removed, and RNA extracted. The RNA was mixed with dextran sulfate of molecular weight 77,000 to 500,000 and injected into untreated C_3H mice either intraperitoneally or subcutaneously. These animals were then challenged with counted tumor cell inocula subcutaneously and statistically significant inhibition of tumor development was noted. Dextran sulfate alone did not result in inhibition of tumor isograft growth. Use of RNA from guinea pigs immunized with normal C_3H mouse tissues was ineffective. Treatment of the active RNA preparations with RNAase again abrogated the response. Specificity for the immunizing tumor was shown and that immune RNA made to one murine sarcoma mediated immunity to that sarcoma but not to a second sarcoma, whereas immune RNA from guinea pigs immunized to the second sarcoma mediated immunity to the second sarcoma but not to the first.

Hartley guinea pigs were then immunized with the benzpyrene-induced sarcoma of Fisher rats, RNA was extracted from the lymph nodes of spleens and mixed with dextran sulfate, molecular weight 77,000, and injected into the foot-pads of untreated Fisher rats every other day for 10 days. The rats were then challenged with a counted tumor cell inoculum from the Fisher sarcoma. A statistically significant decrease in the incidence of tumor development was noted. Again RNAase treatment of the immune RNA abrogated the response and RNA from unimmunized Fisher rats was ineffective in mediating the response.

Significance to Bio-medical Research and Program of the Institute:

This work represents a model of yet another modality for the immunotherapy of cancer, which, it is hoped, may have certain peculiar advantages. Some of these advantages are (1) it would obviate the necessity for the administration of heterologous or homologous antisera, or leukocytes. Administration of such sera or cells can sensitize to leukocyte isoantigens and may result in toxicity or secondary syndromes. The implications of these studies for the immunotherapy of human cancer are as follows. Since heterologous RNA has been shown to be effective and non-toxic, animals other than human beings could be immunized with tumor tissue and immune RNA extracted from the lymphoid tissues. Alternatively, antigen processing cells such as peritoneal macrophages or lymph node cells from such animals could be exposed, in vitro, to human tumor tissue in tissue culture, and immune RNA could then be extracted. Such RNA might be administered to patients by direct injection by mixing the RNA with dextran sulfate. Dextran sulfate is available for clinical use in humans in clinical grades of purity. An alternative method will be to collect immunocompetent cells such as peripheral blood lymphocytes from patients by methods such as leukophoresis on the NCI cell separator. These cells could then be incubated with the "Immune" RNA and then returned to the patient from which they were obtained

only a short time previously. Such a model for immunotherapy would offer several theoretical advantages. First, since antigen processing is bypassed, some anergic patients might be benefited if the anergy were due to defects in antigen processing and recognition, and not due to a lack of immunocompetence of effector cells, i.e., lymphocytes. Second, this sort of immunotherapy would obviate the necessity of administering autologous or homologous tumor cells to cancer patients; (2) no adjuvant is required; (3) this model does not require the administration to patients of homologous or heterologous immunocompetent cells. Such cells contain leukocyte antigens to which the patient can be sensitized. In addition, a danger of graft versus host reaction exists. Moreover, immune elimination of such passively transferred immunocompetent cells might make prolonged immunotherapy ineffective. In our model, immune RNA would be injected directly or incubated with the patients' own autologous lymphocytes. The medicolegal problem of the source of donor leukocytes for immunotherapy is eliminated since leukocytes would not have to be obtained from other cancer patients or normal volunteers and no cross-immunization with tumor tissue would be required of other cancer patients or normal volunteers.

Proposed Course of Project:

Efforts are now underway to isolate and characterize the active RNA in these preparations. There is evidence in the literature to suggest that such preparations may contain immunoreactive RNA-antigen complexes. Methods are being set up to attempt to isolate these RNA-antigen complexes by sedimenting our RNA preparations by ultracentrifugation in gradients of cesium sulfate. Isopycnic banding of "Immune" RNA preparations in these gradients may band a unique species of RNA, possibly bound to tumor antigens. Such RNA-antigen complexes have been termed "super antigens" and are vastly more immunogenic than simple antigen alone. Complexes of such RNA with tumor antigens might behave as "super tumor antigens" and their identification, isolation and characterization will be attempted. Further work will be directed towards investigating the direct injection of "Immune" RNA mixed with dextran sulfates of various molecular weights. Dosage schedules must be worked out, the optimum molecular weight of dextran sulfate must be found. The time relationships of injection of the "Immune" RNA with respect to the time of tumor cell challenge must be studied and the specificity of this reaction must be investigated in detail. In this regard, it is important to point out that since nucleic acids are, at best, very weakly antigenic, one would not anticipate that administration of "Immune" RNA preparations would result in sensitization or in immune elimination of the RNA. Better methods must be found for production of "Immune" RNA in larger amounts. The production of immune RNA, in vitro, by the exposure of antigen processing cells to tumor cells, in vitro, will be studied. Better methods must be found for measuring the serologic response of animals treated with "Immune" RNA and methods must be developed for quantitating the immune cytolysis produced by RNA-incubated spleen cells. A modification of the immune cytolysis method of McKhann is

at present being modified for use in quantitating immune cytolysis of spleen cells incubated with immune RNA. This method uses target cells radiolabelled with tritiated thymidine. An attempt will also be made to measure the mediation of immune cytolysis of human tumor cells, in vitro, by human lymphocytes pre-incubated with "Immune" RNA prepared from guinea pigs immunized to human tumors. Experiments are also planned to treat established, progressively growing tumors in rodents with immune RNA mixed with dextran sulfate and with RNA-incubated spleen cells in an attempt to cause slowing of growth or regression of such established tumors.

Publications:

Deckers, P.J., and Pilch, Y.H.: Studies of the transfer of tumor immunity with RNA. Surg. Forum 21: 124-126, 1970.

Pilch, Y.H., and Ramming, K.P.: Transfer of tumor immunity with ribonucleic acid. Cancer 26: 630-637, 1970.

Ramming, K.P., and Pilch, Y.H.: RNA-mediated immune cytolysis to transplantation antigens in vitro. Surg. Forum 21: 267-269, 1970.

Ramming, K.P., and Pilch, Y.H.: Transfer of tumor specific immunity with RNA: Demonstration by immune cytolysis of tumor cells in vitro. J. Nat. Cancer Inst. 45: 543-553, 1970.

Pilch, Y.H., Bard, D.S., and Ramming, K.P.: The role of the regional lymph nodes in the development of host immunological response to tumor. Amer. J. Roentgen. 111: 48-55, 1971.

Pilch, Y.H., and Ramming, K.P.: Immunological enhancement of murine tumor isografts mediated by RNA from lymphoid organs of xenogeneic immunized animals. Transplantation 11: 10-19, 1971.

Articles in Press:

Pilch, Y.H., Ramming, K.P., and Deckers, P.J.: Transfer of tumor immunity with RNA. Israel J. Med. Sciences 7: 246-258, 1971, and In Weiss, D.W. (ed.): Immunological Parameters of Host-Tumor Relationships. New York, N.Y., Academic Press, in press.

Deckers, P.J., Ramming, K.P., and Pilch, Y.H.: Facilitation of tumor growth by syngeneic normal spleen cells mixed with tumor cells in vitro. Cancer, in press.

Pilch, Y.H., Ramming, K.P., and Deckers, P.J.: Transfer of immunity to transplantation and tumor specific antigens by RNA from lymphoid organs of xenogeneic, allogeneic and syngeneic animals. Transplantation Proceedings, in press.

Deckers, P.J., Edgerton, B.W., Thomas, B.S., and Pilch, Y.H.: Tumor immunity in inbred mice with progressively growing tumors - A study of concomitant immunity. Current Topics in Surgical Research, in press.

Deckers, P.J., and Pilch, Y.H.: Transfer of immunity to tumor isografts by the systemic administration of xenogeneic "immune" RNA. Nature, in press.

Ramming, K.P., and Pilch, Y.H.: Transfer of tumor specific immunity with RNA: Inhibition of growth of murine tumor isografts. J. Nat. Cancer Inst., in press.

Deckers, P.J., Edgerton, B.W., Thomas, B.S., and Pilch, Y.H.: The adoptive transfer of concomitant immunity to murine tumor isografts with spleen cells from tumor-bearing animals. Cancer Research, in press.

Deckers, P.H., and Pilch, Y.H.: RNA-mediated transfer of tumor immunity - A new model for the immunotherapy of cancer. Cancer, in press. Received the James Ewing Society Resident's Award for Research.

Serial No. NCI-3818

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Malignancy Associated Cellular and Serum Abnormalities (Formerly "The Relationship of Lymphocytic Activity and the Immune Mechanism")

Previous Serial Number: Same

Principal Investigator: Paul B. Chretien, M.D.

Other Investigators: None

Cooperating Units: None

Man Years: (July 1, 1970 through June 30, 1971)

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Part I.	In vitro lymphocyte reactivity in a normal population
Part II.	In vitro lymphocyte reactivity in cancer patients
Part III.	The effect of serum from cancer patients on in vitro lymphocyte reactivity
Part IV.	Serum ribonuclease levels in cancer patients

Project Description:

Part I. In vitro lymphocyte reactivity in a normal population

Objectives:

To develop a highly precise method of quantitating in vitro lymphocyte reactivity, and to establish an age-correlated range of normal lymphocyte reactivity by the study of lymphocytes obtained from normal volunteers.

Methods Employed:

The kinetics of in vitro lymphocyte reactivity using PHA as a stimulant and the corporation of tritiated thymidine as determined by liquid scintillation counting was systematically investigated. The methods used by other laboratories were assessed and compared with alternative methods developed in this laboratory. The studies led to the discovery of unacceptable variables in techniques currently employed for the study of in vitro lymphocyte reactivity. It was found that the incorporation of tritiated thymidine by lymphocytes was not maintained at optimum levels for longer than 3 to 4 hours. Beyond this period of progressive decline in optimum incorporation occurred as a result of degradation of the thymidine, and the effects of internal radiation of the radio active label. Thus the labelling periods of up to 18 hours used in other studies were shown to be a potential source of error in experiments lacking internal controls. The use of a single dose of stimulant (in this instance, PHA) inaccurately reflected optimum response when a large number of normal individuals were studied. This led to the development of a dose response curved with a sufficient range of PHA doses so that peak responsiveness by every individual studied was attained. The least variation in the response of a homogenous population of volunteers was seen when the peak response, regardless of the dose of PHA used, were compared. This additional control, seldom used in other published studies, though it greatly increased the number of individual cultures requires for the assay of the response of the single individual, was shown to be essential for the requirements of this study. To determine the period of maximum incorporation of tritiated thymidine, the incorporation was monitored from 0 to 96 hours in a number of normal individuals. Three hourly incorporation intervals in this study showed that the peak rate of tritiated thymidine incorporation occurred between the 50th and 60th hour of culture. This was considerably earlier than the approximately 72-hour interval used in other published investigations. At this later time period cultures were more variable in their response to test antigen because of the inter-relationship of declinic cellular proliferation and increasing cell death. Because of the adverse and variable effect of the associated granulocytes on the incorporation of tritiated thymidine by lymphocytes in culture that was demonstrated in preliminary studies, a technique of rapid separation of lymphocytes from the associated granular sites was developed. This purification technique yields lymphocyte populations with fewer than 5% contamination with other cells and because of its simplicity is suitable for study of large numbers of individuals. Extensive studies of lymphocyte responsiveness utilizing a spectrum of homologous serum concentrations,lymphocyte concentrations, and culture intervals has allowed the selection of the ideal of all these parameters. It was found that with an increasing concentration of homologous serum from a range of 5 to 20% in the cultures, the peak responsiveness of the cultures was unchanged yet the controlled response rose steadily. This led to the selection of a lower concentration of serum than conventionally utilized so as to mainatin a high experimental to control response ratio. Variation in the concentration of lymphocytes from 50,000 to 500,000 per

culture was associated with an increasingly narrow co-efficient of variation with increasing cell numbers, at the expense of a dimunution in peak responsiveness. The study led to the selection of the most optimum cell concentration -- the one with least co-efficient variation and minimum loss of peak responsiveness. As a homologous test serum and supporting media for a population study, a pool of AB serum was collected from a large number of individuals was developed. Each serum contributed to this pool was shown to be devoid of major ABO or HLA antibodies.

Major Findings:

Over 300 normal volunteers ranging in age from 3 to over 80 years have been obtained from a variety of sources. These normal volunteers were carefully screened and those with a history of chronic illness, radiation therapy, or chemotherapy, chronic drug congestion or recent acute illness were excluded. In addition, those found to have abnormalities of blood cell count were also excluded. The study has led to the development of a range of normal in vitro lymphocyte responsiveness correlated with age. The study shows that lymphocyte responsiveness rises from infancy to a peak late in the second decade of life. Their response then rapidly declines during the next two decades, then more slowly declines to the sixth decade where it remains stable. Although a narrow confidence limit of response has been established for the entire population, the maximum variation occurred during middle life. This may reflect a immunologically and biologically more heterogenous population of individuals than those at the extremes of life study.

Significance to Bio-Medical Research and Program of the Institute:

The data derived from this study will serve as a age-correlated range of normal lymphocyte reactivity with which the reactivity of lymphocytes, patients with various malignancies will be compared.

Proposed Course of Project:

The number of normal volunteers of all ages studied in this manner is continuously being expanded. In addition, individuals previously studied or re-evaluated. These additional studies serve as a constant check on the maintainence of homogenous culture condition and comparable results over time intervals. Expansion of this population study to include individuals with a high risk of malignancy, such as heavy smokers, alcholics, and premalignant of the cervix is planned. Such data may allow the selection of a subgroup with abnormal lymphocyte responsiveness that may reflect an increased risk of development of malignancy. Such observations would lead to more frequent scrutiny of such patients in order to detect malignancy at the earlies possible moment. Additional studies of this kind should aide in the detection of epidemialogical factors which predispose to malignancy and other disorders related to abnormalities of the immune mechanism.

Project Description:

Part II. In vitro lymphocyte reactivity in cancer patients

Objectives:

To detect abnormalities of in vitro reactivity of lymphocytes in cancer patients by comparison with normal age-correlated in vitro lymphocyte responsiveness. To determine the effect of definitive treatment of the malignancy on the in vitro lymphocyte response, and to compare the clinical course of the patients with changes in the in vitro lymphocyte response.

Methods Employed:

The methods used in the study of the normal volunteer population described in the preceeding part. The response of lymphocytes to phytohemagluttin was measured by tridiated thymidine incorporation in short term culture. In preliminary experiments, those factors which adversely affect in vitro lymphocyte responsiveness were defined. Chronic or recent ingestion of many drugs, e.g. aspirin, narcotics, and barbituates, recent anesthesia and operation, radiotherapy, chemotherapy, immunotherapy, immunization, acute infections, and organ failure secondary to advanced malignancy all alter in vitro lymphocyte responsiveness. Abnormalities of the sedimentation rate and peripheral white blood cells count, usually associated with obvious associated factors which alter in vitro lymphocyte responsiveness, were also demonstrated to invalidate the studies. Thus, the patients admitted to this study had only relatively early malignancies, and no systemic signs of malignancy or other clinically obvious illness. In vitro lymphocyte responsiveness was determined in AB pool sera rather than autologous sera, to obviate the effect of factors present in the serum of cancer patients that alter lymphocyte responsiveness. The results of this study therefore reflected as nearly as possible alterations in the response of the peripheral lymphocytes that are related to the presence of a localized malignancy. Abnormal lymphocyte responsiveness was defined in relation to the 95% confidence limits for the response of normal volunteers of the same age period. The peak response to phytohemagluttin was compared regardless of the dose of PHA at which it occurred.

Major Findings:

The results of this ongoing study have thus far revealed abnormalities of in vitro lymphocyte responsiveness in patients with early malignancies of all histologic types. Patients with sarcomas had the highest incidence of abnormal in vitro lymphocyte responsiveness. 88% of the patients with this type of malignancy have abnormal in vitro lymphocyte responsiveness. The tumors in these patients were clinically localized and were resected in all instances. None of these patients have thus far developed recurrent or metastatic tumor. Subnormal lymphocyte responsiveness has been demonstrated in 43% of patients with squamous cell carcinoma, 38% of the patients with

melanoma and 29% of patients with adenocarcinoma. In some of these patients, the tumor had metastasized to the regional nodes, but all patients underwent definitive surgery. Thus, patients with advance and clinically incurable malignancy are not included as is the case in some other studies of this type. Some of the patients have developed recurrent or metastatic tumor, but there was no correlation between the score of in vitro lymphocyte responsiveness as normal or subnormal and the development of additional tumor after treatment in the series to date.

Significance to Bio-medical Research and Program of the Institute:

This study attempts to define the relationship between abnormal in vitro lymphocyte responsiveness and the presence of a localized malignancy. Other studies have shown that the in vitro lymphocyte response to PHA correlates with the status of the immune mechanism. Thus, the definition of abnormalities in such a in vitro study is an initial step in the elucidation of those factors leading to the development of malignancy that are related to abnormalities of immunological responsiveness. Such basic knowledge is a rational pre-requisite to the development of means of cancer prevention and more effective methods of treatment. For example, the demonstration of abnormal lymphocyte response in 88% of patients with sarcoma suggest that in such patients, measures that may be taken to reverse the high occurrence of pulmonary metastasis after treatment of the primary cancer (e.g. immunotherapy) would best be approached through treatments which correct subnormal lymphocyte responsiveness.

Proposed Course of Project:

Publication of data. Thus far the studies have been conducted predominantly in those patients admitted to the Surgery Branch of the National Cancer Institute for definitive surgical treatment of localized cancer. A developing study of patients considered clinically cured of their malignancy shows that the in vitro lymphocyte response does not differ from that of the normal population. This data suggest that a study of patients at intervals after definitive treatment of their malignancy should show a response trend that correlates with their clinical course. Such studies may lead to the detection of persistent or recurrent tumor before it is clinically manifest and thus lead to the institution of appropriate treatment at a more advantageous time.

Project Description:

Part III. The effect of Serum from Cancer Patients on In Vitro Lymphocyte Reactivity

Objectives:

To quantitate the effect of serum from cancer patients on the in vitro reactivity of normal lymphocytes. The study is designed to demonstrate the

presence of suppressants of in vitro lymphocyte reactivity in cancer patients' serum.

Methods Employed:

The effect on in vitro lymphocyte reactivity of the serum from blood type matched normal volunteers ranging in age from 3 to over 80 was determined, using PHA as a stimulant as described in Part I. Sera from over 300 normal volunteers was studied in this manner. The reactivity of normal lymphocytes in these normal homologous sera was constant, regardless of the age of the serum donor, and was almost identical to the reactivity of normal lymphocytes in the AB pooled sera described in Part I. With the "normality" of the AB sera relative to its effect on normal lymphocyte reactivity thus established, lymphocytes from normal blood bank volunteers were cultured simultaneously in the sera from cancer patients and the AB pooled serum. The reactivity of normal lymphocytes in the two sera was compared.

Major Findings:

After establishing that normal lymphocyte reactivity in homologous sera from normal persons of the same blood type as the lymphocyte donor was the same as the reactivity in the AB pooled serum, the AB pooled serum thereafter served as a constant criteria of lymphocyte reactivity in homologous sera. A suppressive effect of the sera from cancer patients on normal lymphocyte reactivity was established by this criteria. Thus far, the effect of sera from cancer patients on normal lymphocyte reactivity appears to best correlate with the histologic classification of the tumor. The serum of 50% of the patients with squamous cell carcinoma suppressed normal lymphocyte reactivity below the level of the 95% confidence limits for the effect of homologous serum on lymphocyte reactivity as previously defined. The serum of 13% of patients with melanoma, 25% of patients with adenocarcinoma, and 20% of patients with sarcoma had a like effect on normal lymphocyte reactivity. In general, a higher dose of PHA was required to attain peak responsiveness of normal lymphocytes in the sera that suppressed lymphocyte reactivity than was required for peak responsiveness of the same lymphocytes in normal homologous sera.

Significance to Bio-medical Research and Program of the Institute:

A means by which host-immune responsiveness to malignancy may be impaired may be through humoral suppression of lymphocyte reactivity. The findings in this experiment may reflect the presence of such suppressants of immune responsiveness. The further identification of the factor accounting for the suppressive affect on normal lymphocyte reactivity in these patients may therefore lead to a further clarification of the mechanisms that facilitate the development and growth of malignancy and lead to more effective means of tumor irradiation.

Proposed Course of Project:

Further investigations will be directed toward the precise identification of the suppressant of lymphocyte reactivity in the serum of cancer patients. Investigations by others have shown that the serum of cancer patients contains increased levels of alphaglobulins and new alphaglobulin species; and parallel experiments show that alphaglobuins in high concentration, both *in vivo* and *in vitro*, suppresses immune responsiveness. Further investigations will attempt to determine whether the observations made in this experiment are due to alterations in the alphaglobulin fraction of cancer patients. Also, in this ongoing study, patients will be studied at intervals after definitive treatment of their cancer in an attempt to correlate the sera suppressive effect on lymphocyte reactivity with the patients clinical course. Such correlation should allow early isolation of those patients that have residual tumor and thus lead to institution of treatment at an earlier time. In addition, studies on the sera of patients exposed to carcinogens and those with premalignant lesions may lead to the isolation of a high risk group relative to cancer development.

Project Description:

Part IV. Serum ribonuclease levels in cancer patients

Ojectives:

To correlate abnormalities of serum ribonuclease levels in cancer patients with other abnormalities of the immune mechanism. The demonstration by others of a suppressant effect on lymphocyte reactivity *in vitro* and immunological reactivity *in vivo* by ribonucleases indicated a possible relationship between serum ribonuclease levels and the suppressive effect of normal lymphocyte reactivity demonstrated in the sera of cancer patients.

Methods Employed:

A technique for assay of serum alkaline RNAase levels was developed. The accuracy of the assay is monitored by determination of the ribonuclease activity of a constant test AB pooled serum specimen with each determination of the ribonuclease activity in other sera. The variation in the ribonuclease assay of the AB pooled sera is less than $\pm 3\%$. The serum ribonuclease level of over 200 individuals sera has been determined, and from this, an age-correlated range of normal serum ribonuclease level has been established. This data shows that serum ribonuclease levels are lowest in infancy, and rise progressively to the end of the second decade. From the third to the eighth decade, the mean serum ribonuclease level is constant, however, the 95% confidence limits for the range of normal activity is greatest in the middle decades.

This far, the sera from over 60 patients with various malignancies has been assayed for serum alkaline ribonuclease levels. The incidence of elevated

ribonuclease levels thus far appears to correlate with the histologic classification of the malignancy. The incidence of elevated serum ribonuclease levels was highest in patients with squamous cell carcinoam (50%) and adenocarcinoma (67%). Elevated serum ribonuclease levels were less often found in patients with melanoma (20%) and sarcoma (13%). It may be pertinent that the serum of 50% of patients with squamous cell carcinoma also suppresses normal lymphocyte reactivity. Sufficient number of patients have not been studied thus far, however, to define a relationship between the suppression of lymphocyte reactivity in these patients and the serum ribonuclease levels. It is also of interest that elevated serum ribonuclease levels occurred less often in patients with sarcoma, yet it is in patients with this histologic type of malignancy that in vitro lymphocyte reactivity in homologous serum was most often abnormal (88%). It thus appears that the suppressed response of lymphocytes from patients with sarcoma is not related to elevated serum ribonuclease levels. This correlation is pertinent to the experimental demonstrations by others of suppression of lymphocyte reactivity by ribonucleases.

Significance to Bio-medical Research and Program of the Institute:

A major goal of this ongoing study is the determination of the relationship between elevated serum ribonuclease levels that occur in some cancer patients and other serum and cellular alterations also being demonstrated. If the suppressive effect of the sera of some cancer patients on normal lymphocyte reactivity is related to elevated serum ribonuclease levels a mechanism by which host-immune responses are suppressed is suggested. The clarification of the mechanisms by which host-immune responsiveness is altered facilitates the development of improved methods of cancer treatment. A more obvious importance of the definition of those patients with elevated serum ribonuclease levels is related to possible clinical attempts to increase host-immune responsiveness by the transfer of immune RNA. In such patients, a major deterent to successful transfer would be the destruction of the RNA by elevated serum ribonuclease levels.

Proposed Course of Project:

The increased serum ribonuclease levels in certain cancer patients may reflect the presence of new proteins whose significance may be of greater importance than the associated ribonucleases. The isolation and identification of the proteins with which the serum ribonucleases are associated will be the next phase of this study period.

Publications:

Whang-Peng, J., Chretien, P.B., and Knusten, T.: Polyploidy in malignant melanoma. Cancer, 25: 1216-12237, 1970.

Sample, W.F., Gertner, H.R., Futrell, J.W., and Chretien, P.B.: Suppression of in vitro reactivity of lymphocytes from normal persons by serum from carcinoma patients. Surg. Forum 21: 116-118, 1970.

ARTICLES IN PRESS

Sample, W.F. and Chretien, P.B.: Thymidine kinetics in human lymphocyte transformation: Determination of optimal labelling conditions. Journal for Clinical and Experimental Immunology. In press.

Sample, W.F., Gertner, H.R., Chretine, P.B.: Inhibition of phytohemagluttin induced in vitro lymphocyte transformation by serum from patients with carcinoam. JNCI. In press.

Serial No. NCI-3819

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Cancer Immunology and Immunotherapy in Laboratory Animals

Previous Serial Number: Same

Principal Investigator: George H. Myers, Jr., M.D.

Other Investigators: D.L. Morton, M.D., N.L. Albright, M.D., J.W. Futrell, M.D., M.S. Soloway, M.D., and R.A. Malmgren, M.D.

Cooperating Units: Tumor Immunology Section, Surgery Branch, NCI, and Pathologic Anatomy Branch, NCI

Man Years: (July 1, 1970 through June 30, 1971)

Total:	3
Professional:	2-1/2
Other:	1/2

Project Description:

Part I. Intradermal Immunization against Transplantable Tumors in Inbred Mice.

Part II. The Effect of Neuraminidase on the Immunogenicity of Viral and Chemically Induced Tumors in Mice.

Part III. The Effect of Immunosuppression and the Mammary Tumor Virus on the Growth of Transplanted Mouse Mammary Tumors.

Part IV. Search for a Humoral Anti-tumor Antibody in the Sera of Guinea Pigs and Mice Undergoing Immunotherapy.

Part V. The Influence of the Regional Lymphatics on Tumor Immunity.

Part VI. The Immunogenicity of a Murine Renal Adenocarcinoma.

Part VII. Production of Bladder Tumors in Inbred Mice and a Study of their Immunologic Properties.

Project Description:

Part I. Intradermal immunization against transplantable tumors in inbred mice.

Objectives:

It has been possible to prevent the growth of methylcholanthrene induced sarcomas in isologous guinea pigs by the intradermal immunization with viable tumor cells. This study was designed to ascertain whether or not it is possible to significantly inhibit the growth of mouse tumors, which have been inoculated intramuscularly into isologous hosts by the concomitant intradermal immunization with the same tumor.

Methods Employed:

Using inbred mice an attempt has been made to evaluate the effects of immunotherapy with two tumor systems. A methylcholanthrene induced sarcoma in C57BL/6N mice was chosen as an example of a carcinogen induced tumor, and a spontaneous mammary tumor from a C3H/HeN mouse was chosen as an example of a viral induced tumor. The minimal number of tumor cells which will produce palpable tumors is greatest via the intradermal route. The reason for this is thought to be due to the greater immunoreactive capabilities of the skin as compared to the subcutaneous and intramuscular sites. For this reason the intradermal route was chosen for immunotherapy. After a known tumor producing cell inocula is injected intramuscularly into the hind leg of isologous hosts, a non-tumor producing inocula of live tumor cells is injected intradermally into several sites of the same animal. A corresponding group of animals will receive intradermal injections of irradiated tumor cells.

Major Findings:

Intradermal immunization with irradiated tumor cells has not been effective in preventing the growth of tumor. However, immunotherapy against tumor cells injected intramuscularly is being accomplished by the concomitant intradermal immunization with viable tumor cells, but only in the case of the most antigenic methylcholanthrene induced sarcoma. So far no inhibition of the viral induced mammary carcinoma has occurred. The effects of intradermal immunization in the mice with the methylcholanthrene induced sarcoma is less pronounced than in the guinea pig.

Significance to Bio-medical Research and Program of the Institute:

Further studies in experimental animals are necessary before immunotherapy can be applied to human cancer. Preliminary studies suggest that the presence of small numbers of tumor cells can be prevented from growing by the intradermal immunization with live tumor cells. Although immunotherapy

of cancer may not be of benefit as a primary form of treatment, it may become useful as a supplement to the more conventional forms of treatment, such as surgery and chemotherapy.

Proposed Course of Project:

These studies have been continued using other methylcholanthrene-induced tumors in other animals to further evaluate the effectiveness of intradermal immunotherapy, both as a form of primary treatment as adjunct to surgery and/or chemotherapy.

Project Description:

Part II. The effect of neuraminidase on the immunogenicity of viral and chemically induced tumors in mice.

Objectives:

It has been suggested by other authors that the effective antigenic expression by some types of tumor cells may be inhibited by the presence of sialic (N-acetyl neuraminic) acid on the cell periphery. The treatment of several "non-specific" transplantable mouse tumors with neuraminidase has resulted in an apparent increase in their immunogenicity. Little of the information so far obtained has been related to the growth and development of autochthonous tumors and their antigenicity in isologous hosts. This project was undertaken to study the effects of neuraminidase on the immunogenicity of both a chemically and a viral induced tumor in isologous hosts.

Methods Employed:

A methylcholanthrene induced sarcoma in C57BL/6N mice is being used as an example of a carcinogen induced tumor, and a spontaneous mammary tumor from a C3H/HeN mouse as a viral induced tumor. Tumor cells are incubated with the enzyme neuraminidase which has been obtained from cultures of Vibrio cholerae. Following incubation the tumor cells are resuspended in media and injected at various concentrations into isologous animals. When tumors fail to grow, the animals are rechallenged intramuscularly with a known tumor producing inocula and their immunity measured by a low incidence of tumor development, a delay in tumor appearance, slower growth rate, and prolongation of surgical time as compared to control animals.

Major Findings:

When tumor cells are incubated with the neuraminidase at a concentration of 500 units/ml. in 0.05M sodium acetate-acetic acid buffered saline at pH 5.5 and containing calcium ions, there is a loss of viability, determined by dye exclusion studies, of 30 to 50 percent. The acetate buffer alone causes

the same loss of viability. A methylcholanthrene induced sarcoma which usually grows at $1x10^3$ cells failed to grow at $2x10^6$ cells after being treated with neuraminidase plus buffer or with the buffer alone. However, when those animals were challenged, they failed to demonstrate immunity. When the tumor cells were suspended in a buffer at pH 7.0 and then incubated with neuraminidase, there was no alteration of tumor growth even at a low cell dose as compared to untreated cells. A similar experience has been obtained with the viral induced mammary tumor. So far we have not been able to show an increased immunogenicity with either tumor after treatment with neuraminidase.

Significance to Bio-medical Research and Program of the Institute:

If it is true that some tumor cells have a masking of their antigenic expression by sialic acid, and that neuraminidase treatment can increase their antigenicity, then it is conceivable that these treated cells could be used for immunotherapy of cancer. Similarly, it could be that such a sialomucin coating of the cell surface prohibits the host animal from mounting an effective immune response to cancer.

Proposed Course of Project:

The studies which indicate that neuraminidase can increase the immunogenicity of tumors have been done almost exclusively with the Ehrlich, the Landschutz and TA_3 ascites tumors. There has been one report of methylcholanthrene induced sarcomas which suggests an increase in immunogenicity of neuraminidase treated cells when injected intraperitoneally. We are going to continue to study the effects of neuraminidase as a possible adjunct to immunotherapy with particular attention to routes of immunization and comparisons to other forms of immunization. Neuraminidase treated cells will be used to try and produce an effective intradermal immunization against an intramuscular tumor challenge.

Project Description:

Part III. The effect of immunosuppression and the mammary tumor virus on the growth of transplanted mouse mammary tumors.

Objectives:

During the past several years a number of reports have supported the hypothesis that some, and perhaps all, mouse mammary carcinomas induced by the mammary tumor virus are antigenic in syngeneic mice or even in the autochthonous host. However, the presence of the mammary tumor virus markedly alters the results of immunization studies. Sublethal irradiation of recipient mice has been shown to increase the growth of transplanted mammary carcinomas only when the animals were free of the mammary tumor

virus (MTV-free). This project is being designed to study if other methods of immunosuppression similarly alter the resistance against tumor challenge, and to what extent the presence or absence of the mammary tumor virus affects the resistance.

Methods Employed:

Mammary cancers from C_3H/NeN and BALB/c/f mice will be used during these experiments. Both strains of mice will contain sublines of virus-infected and virus-free mice of the same strain. Methods of immunosuppression will consist of total body irradiation, cortisone and rabbit anti-mouse lymphocyte serum. Each subline will be challenged with tumor following immunosuppression. It is assumed that increased resistance against tumor challenge in mice without the mammary tumor virus is on an immunological basis if the various forms of immunosuppression reduce the resistance of the MTV-free mice against tumor challenge, but has little or no effect on those mice congenitally infected with the mammary tumor virus. If immunosuppression actually allows the virus-infected mice to increase their resistance to tumor, then there is a suggestion that acquired immunological tolerance may be partially broken.

Major Findings:

When mice lacking the mammary tumor virus are challenged with syngeneic mammary tumor following immunosuppression the incidence of tumor growth is increased. Animals positive for the mammary tumor virus have not shown increased growth of syngeneic tumor while they are immunologically suppressed and even at times have shown a slightly slower growth than the controls.

Significance to Bio-medical Research and Program of the Institute:

Preliminary observations with total body irradiation suggest that the acquired immunological tolerance of mice infected with the mammary tumor virus may be partially broken. If tolerance proves to be important for effective carcinogenesis by other oncogenic viruses in nature, then other means for breaking this tolerance will be needed if immunotherapy of viral-induced cancer is to be effective.

Proposed Course of Project:

These studies are being repeated and should be completed soon.

Project Description:

Part IV. Search for a humoral anti-tumor antibody in the sera of guinea pigs and mice undergoing immunotherapy.

Objectives:

It has now been shown that guinea pigs can be protected from intramuscular tumor challenge if they undergo immunotherapy by repeated intradermal injections of viable tumor cells as well as antigens prepared from tumor cells. Although similar findings have also been seen in mice the effect of much less striking than in the guinea pig. The results in the guinea pig have prompted other investigators to begin immunizing humans with autologous or homologous tumor cells. Patients so immunized have been found to contain an antibody in their sera which is thought to be specific for the immunizing tumor. The studies performed in guinea pigs have suggested that the results of immunotherapy were due solely to an increase in a cellular or delayed type hypersensitivity response. Until now, guinea pigs so immunized have not been shown to contain an anti-tumor antibody. It is the purpose of this study to see if we can determine, by the complement-fixation technique, an antibody in the sera of guinea pigs who have successfully undergone intradermal immunization against methylcholanthrene induced sarcomas.

Methods Employed:

Inbred guinea pigs of strain 2 and 13 will be used for this study. Methylcholanthrene induced sarcomas designated MCA-A and MCA-25 will be used as the tumors. Immunotherapy will consist of multiple intradermal injections of a subtumor growth inocula following an intramuscular tumor challenge. The animals will be serially bled by intracardiac puncture. Their serum will be stored, until the end of the entire experiment, when all sera will be examined by a modification of the microtiter complement fixation technique for the presence of an anti-tumor antibody.

Major Findings:

Intradermal immunizations, using irradiated and unirradiated tumor cells, have been shown to be effective in preventing an intramuscular tumor growth in syngeneic guinea pigs. The sera of animals so immunized have been found to contain an antibody that reacts with the MCA-A (in this case the immunizing tumor) antigen and does not react with antigens prepared from MCA-25, normal Strain 2 guinea pig fibroblast grown in tissue culture, or normal Strain 2 liver muscle or heart tissues. Sera have also been obtained from animals which were immunized by repeated intradermal injections of media 1640, fetal calf serum, and Gey's solution with 0.25% trypsin. None of these sera gave a positive complement-fixation technique when reacted with the MCA-A.

Significance to Bio-medical Research and Program of the Institute:

These studies have demonstrated for the first time an antibody in guinea pigs immunized against a carcinogen induced tumor. Previous studies that have detected an antibody against a carcinogen induced tumor have been solely confined to the murine system. Our findings seem to correlate well with the findings that have been recorded from human immunizations. This may become an important model for future immunotherapy. This will also make possible a better characterization of the tumor antigens associated with carcinogen induced tumors.

Proposed Course of Project:

These studies will be continued using other tumors in both guinea pigs and mice. Attempts will be made to further characterize this antibody in the antigen involved.

Project Description:

Part V. The influence of the regional lymphatics on tumor immunity.

Objectives:

The regional lymphatics have been shown to play an important part in the rejection of skin allografts. This has been best shown by Barker and Billingham who have created alymphatic skin pedicles on the flanks of guinea pigs. Skin allografts on such pedicles were found to survive indefinitely. Animals with a pedicle graft in place will reject another graft from the same donor in a "first-set" fashion. Not only is the second graft rejected but the first graft, on the pedicle, is also rejected thereby establishing the presence of intact efferent arc of the immune system. We propose to use a similar experimental model to study effects of the regional lymphatics on tumor allografts as well as tumor isografts.

Methods Employed:

Two methylcholanthrene tumors will be used in this study; one designated MCA-A, the other designated MCA-25. Both of these tumors have originated in Strain 2 guinea pigs and will serve both as allografts and isografts. Strain 2 and Strain 13 animals will be used. Alymphatic skin pedicles will be created on the flanks of the animals and these pedicles challenged with various tumor cell doses. This same model will be used to study immunotherapy and adoptive transfer of tumor immunity in the guinea pig.

Major Findings:

Tumor allografts injected into alymphatic skin pedicles have been found to grow to as large as 3 cm. but all eventually regress. The size of the tumor growth in the pedicles is considerably larger than any tumor produced from allogeneic tumor cells in the intact skin. Animals previously bearing a tumor allograft, if rechallenged, demonstrate immunity and this immunity can be transferred to other animals by the use of spleen cells. In contrast tumor isografts in alymphatic pedicles grow to as large as 6 cm. resulting in the death of the animal. This can be shown to occur with a cell-inoculum dose which would never produce a progressively growing tumor in the intact skin. If, however, a pedicle bearing a tumor isograft is excised early in the course of its growth and the animal rechallenged with syngeneic tumor he also demonstrates immunity. These findings suggest that the regional lymphatics are important for the early initiation of tumor growth but that once this tumor obtains a certain size it no longer is under the influences of the local immune system. In spite of this systemic sensitization the animal is unable to reject a tumor when only weak tumor specific antigens are present.

Significance to Bio-medical Research and Program of the Institute:

Recently a few clinicians have been advocating leaving the local lymph nodes in place during cancer surgery in order to preserve the patient's immune defenses. Our findings would suggest that once a tumor is large enough to be clinically detected the patient has already been sensitized systemically and would not benefit by leaving the local lymph nodes intact since many of these may contain metastatic tumor.

Proposed Course of Project:

Further investigations will be carried out using this animal model system in hopes that a better understanding of the regional lymphatics can be obtained.

Project Description:

Part VI. The immunogenicity of a murine renal adenocarcinoma.

Objectives:

Renal adenocarcinoma or hypernephroma is one of the few tumors that have been known to undergo spontaneous regression in humans. In addition, the often benign and bazaar clinical course associated with this tumor suggest that it may be under immunologic control. The absence of a proper animal tumor model system has previously prevented detection of tumor specific antibodies associated with any renal cell carcinoma. The purpose of this

study is to determine if a renal cell carcinoma which arose in an inbred strain of mice, can be shown to contain tumor specific antigens.

Methods Employed:

A renal cell carcinoma which arose in a Balb-C mouse which had received an injection of a cell-free extract obtained from a human gastric cancer will be studied. Once the growth pattern of this tumor has been obtained, animals will be immunized by temporary tumor growth and amputation. Following amputation, animals will be challenged with a minimal tumor cell dose to determine if an immune response exists. Animals who appear to be immune to rechallenge will also be challenged with other syngeneic tumors, as well as normal skin to determine the specificity.

Major Findings:

Preliminary studies indicate that the tumor being studied does contain tumor specific transplantation antigens which can protect against re-challenge of syngeneic tumors. So far no cross-reactivity has been seen between a Balb-C mammary tumor. Animals rejecting tumor also readily except skin grafts from syngeneic animals.

Significance to Bio-medical Research and Program of the Institute:

If tumor specific transplantation antigens can be demonstrated in this renal cell carcinoma it will be the first demonstration that such antigens can exist in a renal tumor. These findings would give further impetus to the study of human tumors in hopes that here too, we could demonstrate tumor specific antigens. The ultimate outcome of these studies of course, would be eventual immunotherapy against renal cell carcinoma.

Proposed Course of Project:

These studies will be continued in hopes that a model for immunotherapy can be instituted.

Project Description:

Part VII. The production of bladder tumors in inbred mice and a study of their immunologic properties.

Objectives:

Immunologic studies of urinary bladder carcinomas have been hampered by a lack of appropriate experimental model. The canine bladder is sensitive to the carcinogenicity of 2-naphthylamine and 4-aminobiphenyl as well as other compounds regarded as carcinogenic for the bladder in

humans. However, the dog is unsuitable for proper immunologic experiments. Bladder carcinomas have been produced in rats and mice using these above compounds and other compounds but only when used in conjunction with a paraffin or cholesterol pellet. The high incidence of bladder carcinomas produced by the paraffin or cholesterol pellets alone adds an additional carcinogen or the possibility of a synergistic effect with the hydrocarbon-pellet implantation technique. Recently N-[4-(5-Nitro-2 furyl)-2 Thiazolyl] formamide or FANFT, a candidate antimicrobial, 5-nitrofuran drug, has been found to produce a nearly 100% incidence of urinary bladder carcinomas when fed to female and male non-inbred rats and mice. Although FANFT has not yet been studied in inbred rats or mice it appears to be the most suitable compound available to study the immunologic aspects of bladder cancer. It also provides the best experimental model to study the temporal sequence of events in the genesis of urinary bladder carcinomas. In addition, this model may provide the most suitable system yet found to economically study various therapeutic modalities with some assurance that some studies may have relevance to man. Our studies will include two inbred strains of mice to assure tumor production, since mice strains are noted to differ in their susceptibility to chemical carcinogens. In addition, we hope to study the effects of immunosuppression on the production of bladder tumors.

Methods Employed:

Two inbred strains of mice, (1) C57BL/6N and (2) C3H/HeN will be used during this study. Animals will be fed purina laboratory chow containing the appropriate amount of FANFT. Animals serving as controls will receive their normal diet of purine laboratory chow. Others will be given a diet containing the chemical carcinogen and repeated injections of cytoxin or another immunosuppressive agent. Another group will be given cytoxin alone. All animals will be bled periodically to determine their white blood counts. Urine will be collected from all groups at regular intervals, with some of this being put aside for further studies, and a portion of each collection sent for cytologic examination. Skin grafts will be exchanged between the two different inbred strains of animals in order to determine whether or not effective immunosuppression is being obtained. At regular intervals animals will be autopsied and their bladders examined grossly and microscopically for the evidence of tumors as well as a general autopsy for other tumor production. Tumors once found will be transplanted into syngeneic animals for further immunologic studies.

Major Findings:

There is no data yet available since this project is just getting under way.

Significance to Bio-medical Research and Program of the Institute:

Hopefully, this animal model system will produce important information regarding the immunologic aspects of bladder cancer. Once information has been gained from an animal system regarding known chemical carcinogen induced bladder tumors, it may then be possible to intelligently study human bladder cancers with the possibility that some of these tumors are also carcinogen induced.

Proposed Course of Project:

Further investigation.

Publications:

Wood, W.C., Fraley, E.E., and Marshall, J.R.: Postobstructive diuresis complicating relief of lower urinary tract obstruction by uterine leiomyomas. Ob. and Gyn.35: 427-431, 1970.

Fraley, E.E., Ecker, S., and Vincent, M.M.: Spontaneous in vitro neoplastic transformation of adult human prostatic epithelium. Science 170: 540-542, 1970.

Paulson, D.F. and Fraley, E.E.: Chemical evidence for early but unsustained growth in the obstructed mouse kidney. Amer. J. Physiology 219: 872-875, 1970.

Serial No. NCI-3820

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
June 1, 1970 through June 30, 1971

Project Title: Cellular Immunity to Tumor Specific Transplantation Antigens

Previous Serial Number: None

Principal Investigator: A.S. Ketcham, M.D., and A.M. Cohen, M.D.

Other Investigators: J.F. Burdick, M.D., D.L. Morton, M.D., and S.A. Wells, M.D.

Cooperating Units:

Man Years: (July 1, 1970 through June 30, 1971)

Total:	2-1/2
Professional:	1-1/2
Other:	1

Project Description:

Part I. Studies of Cellular Immunity to Tumor Specific Transplantation Antigens in a Guinea Pig Tumor Host System.

Part II. The Human Cellular Response to Malignant Melanoma and to Sarcoma as Studied in vitro with an Immune Cell Mediated Cytotoxicity Assay.

Part III. Demonstration of Common Surface Antigens in Animals and Patients with Malignancy.

Part IV. An Assay of Immune Antibody Cytotoxicity.

Part V. Studies in Animal Tumor Host Systems of the Effect of Various Immunization Schemes and Alterations in the Tumor Cell Surface on Subsequent Tumor Isograft.

Project Description:

Part I. Studies of cellular immunity to tumor specific transplantation antigens in a guinea pig tumor host system.

Objectives:

To study in vitro the splenic lymphocyte response to concomitant tumor growth in a syngeneic Strain 2 guinea pig tumor host system.

To study the effects of various immunization schemes on the development of cellular immunity.

To study the possibility of the development of a humoral efferent enhancing antibody. In addition, to study the development of a central enhancing factor or the development of immunological tolerance to the tumor. The effect of immunization by the intradermal route and by the intrasplenic route will be compared. The effect of altering the surface membranes by neuraminidase, iodoacetate, or by chemical binding of a foreign protein antigen to the tumor surface will be studied.

Methods Employed:

Two noncrossreacting methylcholanthrene induced sarcoma in Strain 2 guinea pigs have been studied. Confirmation of the presence of tumor specific transplantation antigens was confirmed by routine challenge techniques. An assay for in vitro immune cell mediated cytotoxicity was developed over a considerable period of time. This technique involves the labelling of tissue culture target cells with I-125 labelled iododeoxyuridine, a DNA label. After the addition of immune lymphocytes to the tissue culture the loss of radioactivity from the monolayer culture indicated the degree of cellular immunity. The addition of serum to the target cells prior to the addition of lymphocytes can represent an assay for enhancing antibody. Guinea pigs were also studied for the development of delayed cutaneous hypersensitivity, other tumors by making a transplantation antigen extract from tumor cells. Intradermal injections of these extracts elicited a specific delayed cutaneous hypersensitivity reaction that correlated with the immunity of the animal. Alterations in tumor cell surface antigenicity have been undertaken using covalent binding of human gamma globulin to the tumor cell surface.

Major Findings:

The reliability of this in vitro assay of cell mediated immunity was confirmed in a number of experiments. Studies with concomitant tumor immunity with one of the guinea pig tumors demonstrated that five days following the intramuscular injection of a tumor cell inoculum the

animal developed delayed cutaneous hypersensitivity reactions to the tumor extract as well as in vitro demonstration of cell mediated cytotoxicity. This immunity persisted until the twelfth day but by three weeks there was no longer a delayed cutaneous hypersensitivity reaction to the tumor. However, at this time there was a persistent delayed cutaneous hypersensitivity reaction to DNCB demonstrating that the animal in fact, had not become anergic. At the same time skin reactivity to tumor antigen was lost, the in vitro cytotoxicity of the animal's splenic lymphocytes was lost. These data can be explained on the basis of specific tolerance or on the basis of central enhancement.

Significance to Bio-medical Research and Program of the Institute:

There are preliminary reports done by other investigators which suggest the development of central enhancement in certain tumor host systems. Certainly the development of tolerance or the development of enhancing antibody would be of interest. Of pertinence to the clinical situation is that these events occurred before any tumor was palpable and certainly did not represent end-stage phenomenon.

Proposed Course of Project:

These observations in this animal tumor host system will be repeated several times. Attempts will be made to separate the development of enhancing antibody, central enhancement, or the development of specific immune tolerance. Adoptive transfer experiments will need to be done to study the aspect of tolerance. Incubation of lymphocytes overnight prior to the addition of them to the cellular cytotoxicity test may help to answer the question of a central enhancing antibody. The relative degrees of immunity that can be obtained in this system by neuraminidase treatment of tumor cells by iodoacetated treatment of tumor cells or by binding of foreign protein antigen to the tumor cell will be investigated. Plans are under way to use direct intrasplenic inoculations which potentially are an excellent way of immunizing an animal. Repeated intrasplenic injections are quite feasible and humans may represent a variance in clinical immunotherapy.

Project Description:

Part II. The human cellular response to malignant melanoma and to sarcoma as studied in vitro with an immune cell mediated cytotoxicity assay.

Objectives:

The presence and degree of human cellular immunity developing to melanomas and sarcomas and the presence or absence of central and efferent enhancing

antibody is to be studied. The presence of crossreactive tumor specific antigens will be approached with allogeneic cytotoxic lymphocytes.

Methods Employed:

Over the past several years multiple tumor lines and associated skin fibroblasts or muscle lines have been obtained on patients with melanomas and sarcomas by Donald L. Morton, M.D. in the Surgery Branch of the National Cancer Institute. These lines will be used in a study of allogeneic cellular immunity to tumor specific transplantation antigens. A cellular cytotoxicity test is the one employed in our animal experiments with some variation. Peripheral blood lymphocytes are obtained from heparinized blood by a carboneal iron methylcellulos extraction technique which produces 99% pure lymphocytes. The labelling of target cells with I-125 iododeoxyuridine allows for accurate quantitation of these cellular immune responses. Cytotoxicity will be studied using blood from patients who are seen in the surgical clinic for routine followup visits. Presumably, many of these patients will have been cured of their disease some many years ago. Some of these patients will be developing metastatic disease and changes in their cellular immunity during this period of time and the question of presence of central enhancing or efferent enhancing antibody will be investigated. Presence of any crossreacting tumor antigens between the sarcoma and melanoma tissue culture lines will be investigated by cross-application of patients' lymphocytes.

Major Findings:

These studies have just been undertaken and will be carried out for the next year. Preliminary evidence suggests that peripheral blood lymphocyte cytotoxicity can be demonstrated against a number of lines of melanoma and sarcoma in tissue culture.

Significance to Bio-medical Research and Program of the Institute:

It is of great importance to establish the presence of cell mediated immunity towards human malignancies. It has been well established that rejection of allografts is primarily cell mediated and the same seems to be true for most tumors. The absence of any significance in cellular immunity towards human tumors who would render the prospect of any successful immunotherapy rather grim. Of particular importance is to attempt to define an immunological defect that prevents adequate cell mediated destruction of human tumor cells possessing tumor specific antigens. Cellular studies are best suited for the study of enhancing antibody.

Proposed Course of Project:

Over the next six months large numbers of patients along with their family members and large control population will be studied for the presence of cellular immunity towards melanoma and sarcoma tissue culture cells. Attempts will be made to correlate this with stage of disease and associated enhancing antibody formation.

Project Description:

Part III: Demonstration of surface antigen antibody in animals and patients with malignancy

Objectives:

The demonstration of circulating antibody to surface antigens on animal tumors and human tumors. In addition to these being comprised of antibody to transplantation antigen, it also should include all forms of antibody that are generated in response to the surface antigens of tumor cells.

Methods Employed:

Modification of the I-125 antiglobulin technique for monolayer tissue culture cells has been evaluated. This test seems to be quite sensitive and gives high titers of antibodies. Tests are being done to evaluate antibody in hyperimmunized guinea pigs with a methylcholanthrene induced tumor as well as studying human sera for antibody to melanomas, sarcomas, and lung tumors.

Major Findings:

Studies are under way at this time but the test seems very sensitive for the demonstration of surface antibodies.

Significance to Bio-medical Research and Program of the Institute:

This assay represents another attempt to quantitate host immune response to tumor specific transplantation antigens and can be monitored during clinical immunotherapy trials.

Proposed Course of Project:

Total antibody development in concomitant immunity experiments, as well as in human patients, is being undertaken.

Project Description:

Part IV: An assay of immune antibody cytotoxicity.

Objectives:

To study in vitro the efficacy of two new assays for humoral cytotoxicity.

Methods Employed:

Using various animal allograft and tumor isograft systems, two techniques of studying cytotoxicity have been investigated. The first involves labelling target cells with I-125 iododeoxyuridine. Cytotoxic antibody and complement are then added to the system and after incubation overnight, the loss of target cells from tissue culture is monitored by the release of I-125. An additional technique involves using a fluorescent nuclear label, ethidium bromide. This marker has an advantage that it enters only damaged cells similar to trypan blue. By plating cells in small Terasisake wells, the degree of cytotoxicity can be determined under the fluorescent microscope.

Major Findings:

The use of I-125 labelled target cells has been applied successfully to the study of humoral cytotoxicity. Current studies are under way to investigate the feasibility of using the ethidium bromide technique.

Significance to Bio-medical Research and Program of the Institute:

A simple, if possible, automated technique of in vitro humoral cytotoxicity is necessary for screening large numbers of sera from patients with various malignancies. The I-125 labelled target cell technique is fairly simple but the use of completely automated fluorescent technique would be even simpler.

Proposed Course:

Further animal and then human studies are being carried out with these two techniques. It is expected that both of these techniques ought to be considerably simpler than existing cytotoxicity assays.

Project Description:

Part V: Studies in animal tumor host systems of the effect of various immunization schemes and alterations in the tumor cell surface on subsequent tumor isograft.

Objectives:

In several animal tumor host systems, the effect of direct intra-splenic injection of irradiated tumor cells on the immune status of the animal will be studied. There has been relatively little work done on this technique which is, in previos publications, a very effective means of immunization. Certainly this technique could be applicable to human immunotherapy. The effect of iodoacetate treatment of tumor cells on the immunological response following immunization will be evaluated as will the effect of covalent binding foreign protein to the tumor cell surface.

Methods Employed:

Using a methylcholanthrene-induced sarcoma in the Fisher 344 rat, the effect of direct intrasplenic injection of irradiated tumor cells will be studied compared to subcutaneous injection of irradiated tumor cells with and without complete Freund's adjuvant. Under anesthesia, spleens will be placed in a subcutaneous pocket which will allow ready access to multiple intrasplenic injections. Injections will be evaluated under the fluoroscope in terms of spleen portography. Techniques of iodoacetate treatment of tumor cells to modify their immunogenicity as well as neuraminidase treatment of tumor cells has been recently described. Foreign protein, namely human gamma globulin will be bound to rat tumor cells with EDCI. These modified tumor cells will be injected directly into the spleen if this is found to be an efficacious route of immunization.

Major Findings:

The studies which have just been undertaken will be carried out for the next six to 12 months.

Significance to Bio-medical Research and Program of the Institute:

These studies have obvious implications to human immunotherapy. Attempts will be made in these animal systems to relate all work to future possibilities of immunotherapy. Direct intrasplenic injections can be performed with minimal risk to patients.

Proposed Course of Project:

Over the next six months multiple studies with this and other tumor host systems will be evaluated. In addition, if certain modifications of the tumor cells are found to be efficacious, attempts at intradermal immunization in the guinea pig tumor host system will be evaluated.

Serial No. NCI-3808

1. Surgery Branch
2. Tumor Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Host Factors Associated with Implantation and Growth of Cancer

Previous Serial Number: Same

Principal Investigator: Donald L. Morton, M.D.

Other Investigators: F.R. Eilber, M.D., W.C. Wood, M.D., T.C. Hakala, M.D., B.K. Gray, M.D., and J.T. Mehigan, M.D.

Cooperating Units: Laboratory of Immunology, NIAID

Man Years: (July 1, 1970 through June 30, 1971)

Total:
Professional:
Other:

Project Description:

Part I. Immunologic and Virus Studies with Human Neoplasms.

Part II. Cytotoxic Activity in the Serum of Sarcoma and Melanoma Patients.

Part III. Immunologic Reactivity and Prognosis Following Cancer Surgery.

Part IV. Solubilization of Tumor-specific Transplantation Antigens from Methylcholanthrene Induced Guinea Pig Sarcomas.

Part V. Evidence of Interactions Between Humoral and Cellular Immunity In Vivo and In Vitro.

Part VI. Acquired Immunological Tolerance and Carcinogenesis by the Mammary Tumor Virus.

Part VII. Detection of Mammary Tumor Virus (MTV) Antigens by the Antiglobulin Consumption Test (AGCT).

Project Description:

Part I. Immunologic and virus studies with human neoplasms.

Objectives:

Cancer specific antigens have been found in almost every adequately studied viral or carcinogen-induced animal neoplasm. Initially, most of these antigens were demonstrated by their ability to induce tumor-specific transplantation resistance following pre-sensitization of the isogenic host by temporary tumor growth or by pre-treatment with irradiated tumor cells. However, these tumor specific antigens have now been demonstrated by a variety of immunological techniques including cytotoxicity, complement fixation, gel diffusion and immunofluorescence reaction. It is logical to assume that human neoplasms contain cancer specific antigens similar to those found in animal tumors, but there is presently no unequivocal evidence for the existence of such antigens. An initial step in proving the existence of cancer specific antigens in human neoplasms would be the demonstration of antibodies in the serum of cancer patients which react specifically with autologous tumor tissue. The objectives of this study were: (1) to determine whether anti-tumor antibodies could be demonstrated by immunological techniques in the serum of cancer patients, and (2) to determine the antigenic specificity of the tumor antigens from various types of human neoplasms.

Methods Employed:

A variety of immunological techniques have been used in an attempt to detect antibodies in the serum of cancer patients against autologous and homologous tumors. Tumor tissue for these studies were obtained from the surgical specimen as soon as possible after its removal from the patient. Part of the specimen was used to make tumor imprints on glass slides which were stored in a liquid nitrogen refrigerator until used in the direct or indirect immunofluorescent test against acetone-fixed tumor imprints. The remainder of the specimen was preserved by slow freezing in media 1640 containing 10 percent DMSO and storing in the liquid nitrogen refrigerator until used in these studies. When available, normal tissues from the same organ were similarly treated and tested. Tissue cultures are initially from tumors and normal tissues from the same patients to be used in subsequent immunologic studies.

The patient's own serum, homologous serum from other patients with the same neoplasms, and normal sera were tested by the direct and indirect immunofluorescent technique against the acetone-fixed tumor imprints. Similar sera were tested by the complement fixation against extracts prepared from the tumor tissue culture cells.

Major Findings:

A. Human skeletal and soft tissue sarcomas

1. Immunofluorescence studies

Serums randomly selected from 6 patients with osteosarcoma and 2 with chondrosarcoma, 24 immediate family members of the osteosarcoma patients, 4 close associates of 1 osteosarcoma patient, and 25 normal Blood Bank donors have been tested by immunofluorescence techniques for antibodies against 6 different osteosarcomas.

Each patient's serum was found to react not only with his own osteosarcoma but also in 80 percent of tests with the tumors of other patients. The immunofluorescence staining was principally limited to the cytoplasm and cell membrane but faint nuclear staining was also occasionally observed. A high incidence of immunofluorescence antibodies was also demonstrated in tests with serums from members of the immediate family (80 per cent) and the serums from close associates of patient B (91 percent). The tests of serums from normal Blood Bank donors revealed a significantly lower incidence of this antibody (26 percent) than in the serums of patients, their relatives, or close associates. There were not sufficient variations in the sex, blood types, or mean ages of these groups to explain the observed differences in their incidence of antibodies against osteosarcomas.

The results observed with the blocking reaction were similar to those previously reported using the indirect immunofluorescence technique. The unlabelled serums from the osteosarcomas and chondrosarcoma patients as well as the relatives of osteosarcoma patients were able to block the reaction of fluorescein labelled globulins from patients B and C against their own osteosarcoma cells, as well as against the osteosarcoma cells of patients E and F. This indicates that all these serums contain antibodies against the same or a closely related osteosarcoma antigen. The possibility of isoantibodies being responsible for these observations is excluded by the reactivity of each patient's serum with his own osteosarcoma and the blocking of this reaction by the reactive serums of others.

The specificity of these reactions were studied by testing the fluorescein labelled globulins of osteosarcoma patients against other human neoplasms. These sera frequently reacted with sarcomas of other types, particularly liposarcomas, fibrosarcomas, and chondrosarcomas. Reactions were also seen with two of four melanomas tested but not with adenocarcinomas of the breast and colon.

Thus, immunofluorescence studies have revealed a high incidence of antibodies to osteosarcomas in the serums of patients with this disease and their close associates which react with a common antigen (or antigens) in osteosarcomas. The increased incidence of antibodies to osteosarcoma in serums from immediate family members and close associates of osteosarcoma patients suggests the association of an infectious agent with this neoplasm. This agent appears to be capable of infecting close associates of these patients in whom it elicits the formation of antibodies reactive with an antigen (or antigens) in osteosarcomas. The cross reactivity between serums from one osteosarcoma patient and his family members with another patient's osteosarcoma suggests that most osteosarcomas contain the same tumor antigen. Since all animal neoplasms induced by the same virus have been found to contain a common tumor antigen, the presence of a common crossreacting antigen in human sarcomas suggests the possible virus etiology of this neoplasm. The crossreactivity of the serums from osteosarcoma patients with some soft tissue sarcomas and melanomas suggests that these neoplasms may also be associated with an antigenically similar agent.

2. Complement fixation studies:

a. A complement fixation test for the study of human tumor antigens and antibodies has been developed which utilizes selected fresh human serum as a complement source. We have found that guinea pig, rat, baboon, and even some human serum will not serve as a complement source for this reaction. Fresh human umbilical cord serum has been the best source of complement that we have found. This test provides a quantitative, sensitive and reproducible technique for the study of the human tumor antigens and antibodies.

b. Using this complement fixation test, sarcoma specific antibodies have been found in 92 percent of the sera from patients with skeletal and soft tissue sarcomas with a mean titer of 1/98. The incidence of antibody in the serum of normal Blood Bank donors is 21 percent with a mean titer of 1/17. A larger number of patients with other types of malignancies have been tested and found not to contain either a higher incidence or titer of antibody than that found in normal Blood Bank donors.

c. The titer of this antisarcoma antibody shows an excellent correlation with the clinical course of the patient's malignant disease. The antisarcoma antibody titers rise following surgery and remain elevated in those patients who remain free of disease. However, with recurrent disease, the antibody titers fall and drop to 0 in patients with progressively growing sarcomas. Thus, there is a considerable correlation between the level of antisarcoma antibody and the course of malignant disease in a given patient. The antisarcoma antibody has been eluted from sarcoma tissue removed at surgery from two patients. Therefore, in vivo fixation of antibody to tumor does occur as suggested by the relationship of the antibody titer to the clinical course of sarcoma.

d. A sarcoma specific antigen has been found in tissue culture cells derived from several different histologic types of human sarcomas. This sarcoma specific antigen has been found in osteosarcoma, fibrosarcoma, liposarcoma, rhabdomyosarcoma, synovial cell sarcoma, leiomyosarcoma, and mesenchymal sarcoma. This sarcoma specific antigen is not present on normal fibroblasts of sarcoma patients in nonsarcomatous malignancies, normal human organs or fetal organs. The sarcoma specific antigen is cell associated but may be solubilized from tissue culture cells of human sarcomas by a variety of techniques. This soluble antigen is quite stable and has been partially purified by chromatography in polyacrylamide gel electrophoresis. This antigen appears to be a protein since it is partially inactivated by trypsin and fully inactivated by pronase. The partially purified antigen reacts well with specific sarcoma antibody in human sera by complement fixation and has been found to produce delayed cutaneous hypersensitivity reactions in patients with sarcomas but not in patients with other types of malignancies. Hopefully, isolation and characterization of the sarcoma specific antigen will provide us with an immunogen of potential usefulness in immunotherapy of human sarcomas.

3. Relationship of sarcoma specific antigen to an associated viral agent:

The possible relationships of this sarcoma specific antigen to an associated viral agent in human sarcomas has been suggested by the following evidence:

a. There is a common crossreacting sarcoma specific antigen found in all different histologic types of skeletal and soft tissue sarcomas. Since all animal neoplasms induced by this sarcoma virus contain a common viral specific tumor antigen, the presence of a common antigen in human sarcomas suggest a possible viral etiology.

b. The serum of 79 percent of the immediate family members of sarcoma patients have been found to contain antisarcoma antibody to a mean titer of 1/67 which is significantly higher than the antibody titer and incidence in normal Blood Bank donors. This suggested the presence of an infectious agent in human sarcomas that is capable of infecting close associates of sarcoma patients where it elicits the formation of antibodies to a common viral antigen found in human sarcomas.

c. The presence of a filterable agent in human sarcomas was directly demonstrated by the finding that cell-free extracts both from sarcoma tumor tissue and concentrated media from the tissue cultures of human sarcomas were capable of infecting normal human embryonic cells in tissue culture where they induced the formation of a new sarcoma specific antigen so that these antigenically transformed normal cells now reacted with the serums from sarcoma patients whereas the normal uninfected cells have not. Furthermore, absorption of the sarcoma test sera with the sarcoma

tissue culture cells removed all the activity against the original sarcoma culture as well as against the transferred antigen. This indicates that the filterable agent inducing the new antigen was the same as the agent inducing the antigen in the original sarcoma.

d. Virus particles morphologically similar to the "C" type sarcoma virus of the avian, murine, and feline species have been detected in a human liposarcoma. The relationship of these virus particles to the common sarcoma specific antigen remain undefined. In addition, electronmicroscopic studies of this sections from a human chondrosarcoma have revealed many type A viral particles.

Thus, it is evident that the sarcoma specific antigen in skeletal and soft tissue sarcomas is a viral induced antigen. The relationship of the virus inducing this antigen to the etiology of human sarcomas remains to be determined.

B. Malignant melanoma

1. Immunofluorescence studies:

a. Our work with malignant melanomas has been extended. Sixteen melanomas have been studied to the present time. Antimelanoma antibodies have been demonstrated in all the 16 patients whose sera were tested against their own melanomas by the indirect immunofluorescence technique with titers of 1:4 to 1:128. These data suggest that most malignant melanomas contain a common tumor antigen which is immunogenic in patients with this neoplasm.

b. A considerable correlation has been found between the clinical status of malignant melanoma and the presence of antimelanoma antibody. The titer of antibody against autologous tumor tissue has been significantly higher in patients with localized melanoma than in those with widespread disease. Also, in cross-testing of sera against 8 different homologous melanomas the incidence of positive tests has been 75 percent in patients with localized disease as compared to 50 percent in patients with advanced disease.

2. Complement fixation studies:

The immunofluorescence studies with malignant melanoma have been extended using the complement fixation tests. Specific antimelanoma antibody has been found in the sera of melanoma patients against a common melanoma antigen derived from tissue cultures of human melanoma. This antigen is not found in normal fibroblasts, other malignancies, normal adult or fetal human organs in tissue culture. Specific antimelanoma antibody was found in a higher incidence and in a higher titer in patients with localized melanoma and in patients undergoing spontaneous regression of melanomas than

in patients with advanced disease. Furthermore, there was a progressive decline in antimelanoma antibody titers of patients with titers with melanoma as their disease became progressive.

3. Immunotherapy studies:

The immunofluorescence and complement fixation studies suggesting that immunologic factors may play a role in human malignant melanoma has encouraged us to undertake preliminary studies in immunotherapy of this disease using BCG vaccine as an immunologic adjuvant. Eight patients with advanced melanoma and multiple subcutaneous metastases have had BCG directly injected into their metastatic lesions. Some nodules were left uninjected in every patient for control observations. Five of the eight patients have shown some response to this therapy with regression of over 90 percent of the nodules directly injected with BCG. Two patients have also exhibited regression of uninjected nodules. One of these had a complete remission of injected and uninjected nodules and remains free of disease at two years following immunotherapy, but the other patient had only a temporary three months regression of uninjected nodules and eventually expired of his disease. Patients showing a response to immunotherapy had a concomitant four-fold rise in antimelanoma antibody titer as measured by complement fixation, could be sensitized to DNCB and became tuberculin positive following treatment with BCG. No patient who was allergic to DNCB or tuberculin had a significant rise in antibody titer or regression of their metastatic lesions directly injected with BCG. Thus, the preliminary observations indicate that active immunotherapy using BCG as an immunologic adjuvant may have a role in the management of patients with malignant melanoma who are immunologically competent.

Significance to Bio-medical Research and Program of the Institute:

These studies suggest the close association of an infectious agent with some human sarcomas. The identification of this agent and elucidation of its role in the etiology of human neoplastic disease remains to be determined. Obviously, this work has considerable importance to the problem of the virus etiology of human cancer. The presence of a common, sarcoma specific viral induced antigen in all different types of skeletal and soft tissue sarcomas is of considerable importance in terms of immunotherapy and possible prevention of sarcomas with a vaccine. If this sarcoma specific antigen can be isolated and purified, it should be useful as an immunogen for use in active immunotherapy as an adjunct to definitive surgical treatment of sarcomas in an attempt to prevent the development of pulmonary metastases. Also, it is possible that this purified antigen can be used as a vaccine to prevent the development of sarcoma in a susceptible population. The finding that BCG significantly enhances the immunologic response to human melanoma antigens suggest that this agent may be useful for use as an immunologic adjuvant in human immunotherapy.

Proposed Course of Project:

We plan to continue these studies as outlined above.

Project Description:

Part II. Cytotoxic activity in the serum of sarcoma and melanoma patients.

Objectives:

1. The determination of the incidence of cytotoxicity in the serum of patients with these tumors as compared to that of control populations.

2. The evaluation of the specificity of this cytotoxic activity.

3. The evaluation of the level of cytotoxic activity in the course of a patient's disease.

4. The determination of the source of the cytotoxic activity, i.e., is this antibody?

Methods Employed:

Tumor target cells are grown in microtest plates and coated with patients' serum and exogenous complement. The surviving cells are enumerated under a phase microscope.

Major Findings:

1. Antibodies cytotoxic to human sarcoma cells were found in 70 percent of the sera from sarcoma patients and 58 percent of their family members and close associates. Similar antibodies were found in only 8 percent of sera from normal blood bank donors and 10 percent of patients with other types of neoplasms. Sera from four sarcoma patients were found to be cytotoxic to their own autologous cell lines in tissue culture. The level of cytotoxic antibody rises rapidly following immunization with autologous sarcoma cells.

These data suggest the presence of a common cell surface antigen in human sarcomas which renders the cell vulnerable to immune cytolysis by cytotoxic antibody.

2. Cytotoxic antibodies were also demonstrable in the sera of melanoma patients against their own autologous cells in culture. Many of these sera were also cytotoxic to the cell lines derived from other patients' melanomas, but the extent of cross reactivity varied between different cell lines from 75 percent to 25 percent.

Significance to Bio-medical Research and Program of the Institute:

Antibodies have been demonstrated by Morton, Eilber, Malmgren, et al in the serum of sarcoma and melanoma patients directed against specific tumor antigens. These studies indicate that these antibodies are also directed against cell surface antigens or intracellular components. The finding of C^1 dependent cytotoxic activity in the serum of these patients strongly suggests that they have antibody directed against antigens expressed on the cell surface (transplant Ag). This also provides a method of following the immune response in an immunotherapy situation.

Proposed Course of Project:

Present work will be continued.

Project Description:

Part III. Immunologic reactivity and prognosis following definitive cancer surgery.

Objectives:

The purpose of this study was to investigate preoperative patients with localized solid neoplasms in terms of their capacity to display delayed cutaneous hypersensitivity to a variety of skin test antigens and to relate this immunologic reactivity to the subsequent course of their malignant disease.

Methods Employed:

Patients included had neoplasms of the head and neck, skin, breast, colon, female genital tract and sarcomas. Also studied were small groups of patients with benign disease, 5-year survival from treated malignancies, and some patients with spontaneous regression of cancer.

They were tested with a battery of common skin test antigens (Blastomycin, coccidiodin, histoplasmin, mumps, mixed tree molds, Dermatophytin, and PPD) as well as sensitized and challenged with varying doses of 2-4 dinitrochlorobenzene.

Major Findings:

1. The capacity to react to the above antigens was found to correlate closely with the results of cancer surgery. Ninety percent (63/70) of patients that were DNCB positive were found to be free of disease at six months. Whereas 97 percent (39/40) of those that were DNCB negative were found to be inoperable or developed recurrence within six months of definitive surgery.

2. The correlation of impaired immunologic reactivity and early recurrence or inoperability was found to be characteristic of squamous cell carcinoma of the head and neck and the cervix as well as malignant melanoma. However, we have found that soft tissue and skeletal sarcomas do not follow this pattern of reactivity since patients with these neoplasms have good immunologic reactivity to DNCB in the presence of widespread metastatic disease. The explanation for this difference in reactivity is unknown at the present time.

Significance to Bio-medical Research and Program of the Institute:

1. The observation that patients who are unable to develop delayed cutaneous hypersensitivity to DNCB have a high incidence of early recurrence following definitive surgery may be useful prospective information in the management of patients where radical surgical procedures are contemplated.

2. Hopefully, the evaluation of cell-mediated immunologic reactivity will be of aid in planning immunotherapy for the control of malignant disease as active immunization in an immunologically competent host and passive immunotherapy in an anergic host would appear to have the greatest hope of success.

3. The marked difference in immunologic reactivity in patients with skeletal and soft tissue sarcomas as compared to patients with squamous cell carcinomas may have considerable theoretical significance in terms of clues to possible etiology of the neoplasms.

Project Description:

Part IV. Solubilization of tumor specific transplantation antigens from methylcholanthrene induced guinea pig sarcomas.

Objectives:

To determine if water soluble tumor specific transplantation antigens can be extracted and purified from methylcholanthrene induced guinea pig sarcomas.

Methods Employed:

Water soluble extracts were prepared from two different methylcholanthrene induced strain 2 guinea pig sarcomas by sonication followed by centrifugation at 130,000 x g. Soluble extracts have also been prepared by a homogenization and ultracentrifugation according to a previously described technique.

Major Findings:

1. Animals immunized with solicated, water soluble extracts from each tumor proved to be significantly more resistant to growth of the same tumor than were the untreated control guinea pigs or syngeneic guinea pigs immunized with antigen derived from a different tumor.

2. Soluble extracts prepared from these tumors have been found to elicit tumor-specific delayed cutaneous hypersensiti ity reactions in animals immunized with the tumor from which the antigen was prepared. These reactions are specific for the immunizing tumor and do not elicit skin reactions in animals immunized with a different syngeneic tumor.

3. These tumor-specific transplantation antigens have been partially purified with DEAE chromatography and polyacrylamide gel electrophoresis. The purification of these antigens has been evaluated by their ability to elicit delayed cutaneous hypersensitivity reactions in skin of guinea pigs immunized by temporary growth.

Significance to Bio-medical Research and Program of the Institute:

The studies demonstrate that carcinogen induced tumor-specific transplantation antigens can be prepared in a soluble form. It should now be possible to purify and study the chemical nature of these antigens.

Proposed Course of Project:

At the present time, we are attempting to further purify these antigens by means of column chromatography and polyacrylamide gel electrophoresis. It is hopeful that all will be able to obtain a relatively pure antigenic molecule which can then be characterized according to its molecular weight and amino acid composition.

Project Description:

Part V. Evidence of interactions between humoral and cellular immunity in vivo and in vitro.

Objectives:

1. The evaluation of the humoral and cellular components of immunity to carcinogen induced tumors.

2. An investigation of the relationship and possibly competition between these components of tumor immunity.

3. The correlation of in vitro results with an in vivo assay of immunity.

Significance to Bio-medical Research and Program of the Institute:

In vitro evidence has been presented by the Hellstroms that serum from tumor-bearing animals and patients has a detrimental effect on the immune activity of their lymphocytes directed against their tumor cells. If this finding in vitro can be confirmed and correlated with similar results in vivo it will have profound influence on the induction of immunotherapy and evaluation of the components of immunity in cancer patients.

Proposed Course of Project:

The experiments are progressing to correlate the in vitro with the in vivo results described.

Project Description:

Part VI. Acquired immunological tolerance and carcinogenesis by the mammary tumor virus.

Objectives:

Cancer specific antigens capable of eliciting a host immune response which specifically inhibits neoplastic growth have been demonstrated in all viral-induced neoplasms which have been adequately studied. Therefore, natural infection with an oncogenic virus would not be expected to proceed to tumor formation unless this host immune response has been inhibited in some manner. Perhaps the most likely mechanism for immune suppression by tumor viruses in nature is by the induction of specific immunologic tolerance to the viral-induced cancer specific antigen following neonatal infection with the oncogenic virus. The natural history of the mouse mammary tumor virus made this neoplasm an ideal model which could be used to test our hypothesis. The mammary tumor virus is present in high concentration in the milk of mouse strains carrying this virus; therefore, the newborn mouse is exposed to the virus from the moment it begins to suckle from its mother's breast. Its initial exposure is begun at a time prior to the onset of immunologic competence and continues daily until weaning. This situation might be expected to produce immunological tolerance to the mammary tumor virus itself, and perhaps to any viral antigens present on the cell membrane of tumor cells induced by this virus. Our previous work has suggested that mice congenitally infected with the C3H mammary tumor virus become immunologically tolerant as adults to a cancer specific antigen in mammary carcinomas induced by this virus.

Our present objectives are as follows:

1. To determine if acquired immunological tolerance is of importance in the development of mammary tumors in other strains of mice that develop a

incidence of spontaneous mammary adenocarcinomas, such as strain DD and the high mammary tumor sublines of the BALB/c and A-Hen strains.

2. To develop methods of abolishing the acquired immunological tolerance induced in C3H mice by congenital infection with the mammary tumor virus.

3. To determine the antigenic specificity of the mammary tumor viruses from various strains of mice developing a high incidence of mammary adenocarcinomas.

4. To determine whether tumor specific transplantation resistance can be induced against spontaneous mammary tumors in mice congenitally infected with the mammary tumor virus.

Methods Employed:

1. Our experimental method of determining the existence of immunological tolerance to mammary adenocarcinomas consisted of comparing the resistance against challenge with mammary tumor inocula of adult mice congenitally infected with the mammary tumor virus with the resistance to similar inocula of genetically identical mice freed of this virus by foster nursing on sublines of mice not infected with the mammary tumor virus. It is assumed that increased resistance against challenge with mammary tumor found in sublines of mice uninfected with mammary tumor virus is on an immunological basis if a total body irradiation greatly reduces the resistance of virus-free mice against tumor challenge but has little or no effect upon the response of mice congenitally infected with the mammary tumor virus.

2. We have attempted to abolish the immunological tolerance to spontaneous mammary adenocarcinomas exhibited by mice congenitally infected with the mammary tumor virus by the passive transfer of immuno-competent spleen cells from MTV-free nontolerant syngeneic mice. Also, an attempt has been made to change the antigenic specificity of the tumor specific antigen (or antigens) on the mammary tumor cell by coupling the tumor cell with a heterologous antigen by bis-diazotization to heterologous proteins such as human gamma globulin and hemacyanin. It is hoped that these heterologous proteins will act as a hapten to the tumor-specific antigen and thereby change the immunological recognition of the tolerant mouse to the tumor-specific antigen.

3. The antigenic specificity of the mammary tumor virus from various strains of mice is being investigated using sublines of virus infected and virus-free mice from the same strain. The virus-free sublines of mice are immunized with histo-incompatible mammary tumor originating in other strains of mice, sich as the A, DD, RIII, and C3H. The tumor being allogeneic is rejected, but the virus-free mouse is exposed to both the mammary tumor virus and any virus specific tumor antigens in the mammary

carcinoma cell. Serum from the immunized mice is then tested for antibodies against a mammary tumor arising in the virus infected subline of the same strain of mice in which the antiserum was produced. Since the antiserum is tested against isogenic mammary tumor, any serological reaction observed must be related to virus specific or tumor specific antigens in the mammary tumor. Immunological techniques currently being applied to the problem consist of immunofluorescence, quantitative complement fixation and anti-globulin consumption.

4. Attempts were made to induce tumor specific transplantation immunity in C3H/HeN mice congenitally infected with and tolerant to the MTV against spontaneous mammary tumors arising in this strain by: (1) allowing temporary tumor growth followed by excision and (2) immunization with irradiated tumor tissue.

Major Findings:

1. Adult C3H/HeN or C3H x C57 F_1 mice which are congenitally infected with the mammary tumor virus appear to be completely immunologically tolerant to spontaneous mammary adenocarcinoma induced by this virus. Studies with sublines of the BALB and DD strains of mice indicate that immunological tolerance also is produced by neonatal infection with both the DD and C3H strains of mammary tumor virus. Furthermore, DD strain of mammary tumor virus appears to be capable of inducing immunological tolerance to mammary tumors induced by the C3H strain of mammary tumor virus and vice versa. Thus, it appears that acquired immunological tolerance may be of importance in the development of mammary tumors in many strains of mice. However, when C3H x C57BL hybrid mice are foster nursed by A/HeN mice, they are not immunologically tolerant to spontaneous mammary adenocarcinomas induced by the C3H mammary tumor virus. The explanation for this appears to be that the A/HeN mother does not pass the MTV to her nursing young in sufficient quantity to induce complete tolerance since the C3H and A/HeN mammary tumor viruses have been found to share common antigens by the antiglobulin consumption test.

2. The immune tolerance to spontaneous mammary adenocarcinomas exhibited by mice congenitally infected with the MTV can be broken by the passive transfer of immuno-competent spleen cells from MTV-free mice previously sensitized against these tumors.

3. Tumor-specific transplantation immunity could be induced against 5 of 11 different spontaneous mammary adenocarcinomas arising in C3H/HeN mice congenitally infected and tolerant to the MTV. Normal tissues from the same mouse in which the tumor originated would not induce immunity and were not rejected when transplanted into tumor immune mice. Immunity appeared to be individually specific against the tumor used for immunization and extensive cross immunity against other mammary adenocarcinomas was not

observed. This immunity appeared unrelated to the MTV since pretreatment with normal and neoplastic tissues containing the agent did not induce similar resistance against tumor challenge. Thus, spontaneous mammary adenocarcinomas contain tumor specific antigens unrelated to the MTV that are immunogenic in isogenic mice congenitally infected and tolerant to the MTV. The demonstration of non-crossreactive tumor-specific antigens in mammary carcinomas, analogous to the antigens of carcinogen-induced neoplasms represents a new class of antigens in virus-induced tumors.

4. All attempts to increase the antigenicity of spontaneous mammary tumors by coupling the tumor cell to the heterologous antigen have been unsuccessful, with one minor exception. Coupling of mammary tumors to hemolymph resulted in a very slight increase in antigenicity over that obtained by immunization with tumor cells alone.

5. Virus-free mice immunized with repeated infections of histo-incompatible mammary tumors have formed antibodies which react with isogenic mammary tumor cells by immunofluorescence, complement fixation and antiglobulin consumption tests. Most of our data has been accumulated with an antiserum produced in a C3Hf mouse and tested against a C3H/HeN spontaneous mammary tumor. Thus far, every mammary tumor induced by the C3H strain of mammary tumor virus (regardless of whether that tumor arose in a BALB/c C3H, or RIII strain of mice) has been able to absorb the reactivity of the C3Hf antisera against the C3H mammary tumor. Furthermore, lactating mammary tissue from virus infected, but not from virus-free mice has similarly been capable of removing this antibody activity. Therefore, it would appear that the antibodies being detected by these tests are antibodies against the mammary tumor virus itself or a virus specific antigen produced in both normal and tumor cells following infection with the virus.

Significance to Bio-medical Research and Program of the Institute:

Further studies will be necessary to evaluate the importance of acquired immunological tolerance in the natural history of other oncogenic viruses. However, if the ability to induce immunological tolerance proves to be an important prerequisite for effective carcinogenesis by oncogenic viruses in nature, then effective immunotherapy of virus-induced cancer will depend on the development of methods for braaking this acquired immunological tolerance to the viral-induced cancer antigens, rather than upon the production of cancer vaccines. A vaccine against breast cancer in C3H mice would not be expected to either prevent tumor development or retard its growth, since the mice that will develop breast cancer cannot recognize the tumor antigen as foreign because they are already immunologically blind to this antigen. However, the demonstration of non-crossreacting tumor-specific antigens in these spontaneous mammary tumors represents a new class of antigens in viral-induced neoplasms. It would appear that the major defense against mammary tumor genesis in mice congenitally infected with the MTV is their immune response against this weaker group of tumor specific antigens.

Proposed Course of Project:

We feel that this work is of enormous importance to our understanding of the mechanism for viral oncogenesis in nature. Therefore, we plan to continue work on this project along the lines indicated above.

Project Description:

Part VII. Detection of mammary tumor virus (MTV) antigens using the antiglobulin consumption test (AGCT).

Objectives:

1. To determine if the highly sensitive antiglobulin consumption test would offer a sensitive method for detecting murine anti-MTV antibody and MTV antigen.

2. To determine if MTV from different mouse strains share a common viral antigen.

Methods Employed:

C3Hf/HeN and BALB/c (MTV-) mice were immunized with RIIIfC3H (MTV+), DD/He, A/He, DBA/2JN and RIII sera from these immunized mice were reacted against histo-compatible BALB/cfC3H or C3H/HeN (MTV+) spontaneous mammary tumors by means of the AGCT. The specificity of the reaction was investigated by absorbing the antisera with various MTV+ and MTV- normal and neoplastic tissues.

Major Findings:

1. The anti-sera reacted against all five C3H mammary tumors tested. Absorption with C3Hf (MTV-) and BALB/c (MTV-) breast tissue did not remove the activity. Absorption with carcinogen-induced tumor (MTV-) also failed to remove the activity. However, absorption with C3H/HeN (MTV+) breast tissue, and C3H (MTV+) RIIIfC3H (MTV+) and DDfC3H (MTV+) mammary tumors significantly reduced the anti-MTV activity of the anti-sera. These results indicate that the antibody detected here is reacting against the MTV itself or a cell surface, MTV-induced antigen present in normal and neoplastic cells.

2. The correlation of impaired immunologic reactivity and early recurrence or inoperability was found to be characteristic of squamous cell carcinoma of the head and neck and the cervix as well as malignant melanoma. However, we have found that soft tissue and skeletal sarcoma do not follow this pattern of reactivity since patients with these neoplasms have good immunological reactivity to DNCB in the presence of widespread metastatic disease. The explanation for this difference in reactivity are unknown at the present time.

Significance to Bio-medical Research and Program of the Institute:

These studies indicate that MTV-free mice are capable of forming humoral antibodies against the MTV which can be detected by the AGCT. The AGCT may prove useful in the detection of other viral antigens.

Proposed Course of Project:

At the present time, we are utilizing this method of antigenic analysis to determine whether the MTV carried by other mouse strains are antigenically similar.

Publications:

Morton, D.L., Eilber, F.R., Malmgren, R.A., and Wood, W.C.: Immunological factors which influence response to immunotherapy in maligant melanoma. Surgery 68: 158-164, 1970.

Eilber, F.R., and Morton, D.L.: Demonstration in sarcoma patients of antitumor antibodies which fix only human complement. Nature 225: 1137-1138, 1970.

Morton, D.L., Eilber, F.R., Malmgren, R.A., and Cooke, K.O.: Evidence for a virus in human sarcomas. Proceedings, Fourth International Conference for Comparative Leukemia Research, pp. 754-760, 1970.

Eilber, F.R., and Morton, D.L.: Immunologic studies of human sarcomas: Additional evidence suggesting an associated sarcoma virus. Cancer 26: 588-596, 1970.

Morton, D.L., Eilber, F.R., Joseph, W.L., Wood, W.C., Trahan, E., and Ketcham, A.S.: Immunological factors in human sarcomas and melanomas: A rational basis for immunotherapy. Ann. Surg. 172: 740-749, 1970.

Eilber, F.R., and Morton, D.L.: Immunologic and immunotherapeutic studies with human sarcomas. Surg. Forum 21: 127-129, 1970.

Joseph, W.L., Melewicz, F., and Morton, D.L.: Spontaneous development of mammary adenocarcinoma following prolonged immunosuppression in the dog. Cancer Res. 30: 3606-3608, 1970.

Wood, W.C., and Morton, D.L.: Microcytotoxicity test: Detection in sarcoma patients of antibody cytotoxic to human sarcoma cells. Science 170: 1318-1320, 1970.

Holmes, E.C., and Morton, D.L.: Demonstration by the antiglobulin consumption test with murine antisera of common antigens in tissues infected with the mammary tumor virus from different mouse strains. J. Nat. Cancer Inst. 46: 253-260, 1971.

Holmes, E.C., Morton, D.L., Schidlovsky, G., and Trahan, E.: Cross-reacting tumor specific transplantation antigens in methylcholanthrene induced guinea pig sarcomas. J. Nat. Cancer Inst. 46: 693-700, 1971.

Joseph, W.L., Morton, D.L., and Adkins, P.C.: Variation in tumor doubling time in patients with pulmonary metastatic disease. J. Surgical Oncology 3: 143, 1971.

Eilber, F.R., Holmes, E.C., and Morton, D.L.: Immunotherapy experiments with a methylcholanthrene-induced guinea pig liposarcoma. J. Nat. Cancer Inst. 46: 803-808, 1971.

Wood, W.C., and Morton, D.L.: Host immune response to a common cell surface antigen in human sarcomas: Detection by cytotoxicity tests. New Eng. J. Med. 284: 569-572, 1971.

Morton, D.L., Holmes, E.C., Eilber, F.R., and Wood, W.C.: Immunological aspects of neoplasia: A rational basis for immunotherapy. Ann. Intern. Med. 74: 587-604, 1971.

Holmes, E.C., Morton, D.L., and Ketcham, A.S.: Parathyroid carcinoma: A collective review. Ann. Surg. 169: 631-640, 1969.

Kagan, A.R., Morton, D.L., Hafermann, M.D., and Johnson, R.E.: Evaluation and management of radiation-induced pericardial effusion. Radiology 92: 632-634, 1969.

Morton, D.L., Kagan, A.R., Roberts, W.C., O'Brien, K.P., Holmes, E.C., and Adkins, P.C.: Pericardiectomy for radiation-induced pericarditis with effusion. Ann. Thorac. Surg. 8: 195-208, 1969.

Articles in Press:

Morton, D.L., and Eilber, F.R.: Prognostic significance of cutaneous anergy for the surgical treatment of solic neoplasms. In Hall, T.E. and Foye, L. (eds.): Prognostic Factors in Human Cancer. National Cancer Institute, Monograph No. 34.

Malmgren, R.A., and Morton, D.L.: Viral and immunologic studies of human neoplasms: A review. Pathobiology Annual, Volume I.

Morton, D.L., Eilber, F.R., and Malmgren, R.A.: Immune factors in human cancer: Malignant melanomas, skeletal and soft tissue sarcomas. Progress in Experimental Tumor Research, Volume 14.

Morton, D.L.: Immunological studies with human neoplasms. J. Reticulo-endothelial Society.

Serial No. NCI-3815

1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tissue and Organ Transplantation

Previous Serial Number: Same

Principal Investigators: D.L. Morton, M.D., W.L. Joseph, M.D., and P.B. Chretien, M.D.

Other Investigators: A.S. Ketcham, M.D., Y.H. Pilch, M.D., E.C. Holmes, M.D., B. Kahan, M.D., R. Reisfeld, M.D., K.P. Ramming, M.D., F.R. Eilber, M.D., and J.W. Futrell, M.D.

Cooperating Unit: Laboratory of Immunology, NIAID

Man Years: (July 1, 1970 through June 30, 1971)

Total:	8 1/2
Professional:	3
Other:	5 1/2

Project Description:

Part I. Lung Transplantation in the Baboon.

Part II. Lung Transplantation in the Dog.

Part III. Quantitation of Immunosuppressive Therapy in the Treatment of Allograft Rejection.

Part IV. The Effect of Phytohemagglutinin on in vivo Immune Responses in the Guinea Pig.

Project Description:

Part I. Lung Transplantation in the Baboon.

Objectives:

1. To see if the autotransplanted lung could assume immediate and total respiratory support without an elevation in PA pressure.

2. To see if the baboon was a better model for physiologic study of lung transplantation.

Methods Employed:

Left lung autotransplants and immediate contralateral PA ligations were performed in 30 baboons. Serial blood gases and right heart catheterizations were performed over the entire course of the experiment.

The tolerance of the lung to ischemia was studied in 43 baboons.

Major Findings:

Twenty-four baboons are alive six to twelve months following operation; six died in the immediate postoperative period. There was a temporary drop in Po_2 in the early postoperative period which returned to normal by two weeks. Fifty percent of the survivors had a transient increase in PA pressure which returned to near normal levels by 14 days. There was little change in the pressure of the other twelve long-term survivors.

The baboon lung is capable of providing immediate and total respiratory function after four hours of inflated normothermic ischemia. Five hours of ischemia was also tolerated by the addition of either continuous ventilation or external surface cooling at 10° C.

Significance to Bio-medical Research and Program of the Institute:

The autotransplanted lung is capable of providing immediate and complete respiratory support. This can be accomplished without a significant increase in PA pressure. A better model for studying the physiologic aspects of lung transplantation has been developed, free of the complications usually seen in the dog with consistent long-term survival.

There is a great potential need for lung transplantation among patients with chronic pulmonary emphysema, pulmonary fibrosis, children with cystic fibrosis, and patients with severe pulmonary disease who develop carcinoma of the lung and need resectional surgery. Therefore, the development of a successful lung transplant program would have an almost unlimited clinical application.

Proposed Course of Project:

We plan to continue this project in the manner outlined above in the hope that the laboratory program will be sufficiently successful and will warrant clinical application.

Project Description:

Part II. Lung Transplantation in the Dog.

Objectives:

To develop an inbred group of dogs whose tissue typing characteristics would be similar to human family conditions such that experimental lung transplantation could be performed under conditions that would be similar to the human aspects.

Methods Employed:

Ten inbred (third generation) Labradors were purchased for inbreeding and further experimental lung transplantation.

Major Findings:

Twenty-five animals as a result of inbreeding are now available. Part of this group has been tissue typed for eventual transplantation.

Significance to Bio-medical Research and Program of the Institute:

An inbred, hearty experimental model for lung transplantation has been developed which will be similar to human conditions.

Proposed Course of Project:

We plan to continue this project in the manner outlined above in the hope that the laboratory program will be sufficiently successful and will warrant clinical application.

Project Description:

Part III. Quantitation of Immunosuppressive Therapy in the Treatment of Allograft Rejection.

Objectives:

At present there is no uniform and reliable method to determine the proper immunosuppressive regimen in the treatment and prevention of allograft rejection. The ability to manifest delayed cutaneous hypersensitivity to dinitrochlorobenzene (DNCB) following skin grafting in dogs was evaluated as a possible method to quantitate drug therapy.

Methods Employed:

Dogs were sensitized and challenged to appropriate doses of DNCB. Skin allografts were then placed on dogs and various methods of immunosuppression were tested. During therapy these animals were challenged to DNCB and the changes in their responses evaluated.

Major Findings:

In six dogs with allografts and no immunosuppression, there was no change in DNCB reactiveness. When proper immunosuppressive was used, the skin grafts survived for 21 days before they were rejected. During this time, the animals failed to demonstrate DNCB sensitivity until their skin grafts rejected. In six additional dogs with skin allografts, DNCB sensitivity was not shown until the drug therapy was reduced to allow the skin grafts to reject.

Significance to Bio-medical Research and Program of the Institute:

It appears that at least in skin allografts, when drug therapy is given in sufficient dosage to prevent rejection, previously sensitized animals will not react to a DNCB challenge. However, when the drug therapy is not sufficient and the grafts reject, DNCB reactiveness returns. It appears that DNCB reactiveness correlates with adequate immunosuppression and allograft rejection.

Proposed Course of Project:

We plan to continue these studies using organ transplantation of lungs and kidneys to determine whether DNCB reactivity correlates with the degree of immunosuppression as indicated by organ rejection as well as skin allograft rejection.

Project Description:

Part IV. The Effect of Phytohemagglutinin on in vivo Immune Responses in the Guinea Pig.

Objectives:

This is part of a systematic investigation of the effects of phytohemagglutinin (PHA) on immune responses in vivo. Previous studies have assessed the effects of PHA on immunosuppressive regimens in dogs. In the present study, the effect of PHA on the immune response to bovine gamma globulin (BCG) tuberculin (DCG) and skin allografts in guinea pigs was determined.

Methods Employed:

Hartley strain guinea pigs were used to study the effects of PHA on the immune responses to BGG and BCG. The animals were immunized with a subcutaneous injection in the nuchal region with a 2% solution of BGG containing heat-killed Microbacterium Bovis (BCG) at a concentration of 5 mg. per ml. The animals were bled by cardiac puncture at 1, 2, and 3 weeks after immunization, and serum antibody titer to BCG assayed by the tanned red cell hemogluttination technique. Titer was expressed as the reciprocal of the highest dilution of serum showing 4+ agglutination macroscopically. A portion of the animals immunized as above were challenged with one microgram of tuberculin-D at 2, 3, 5, 7, and 9 weeks after immunization. The mean diameter of erythema was measured 24 hours after this challenge. Inbred guinea pigs, Strain NIH 2 and 13, were used in the study of skin allograft survival. Full thickness circular skin grafts, 3.5 cm. in diameter, were exchanged orthotopically between Strains 2 and 13 guinea pig pairs. On day 8, 10, 12, and 14, the eliptical biopsies were taken at the periphery of the graft. The allograft survival time was determined by microscopic examination of histologic sections prepared from the biopsies. The criteria for rejection was a uniform mononuclear cell infiltrate in the graft dermis. All of the experimental animals, received 1 ml. of phytohemagglutinin-P (PHA) intraperitoneally daily, commencing two days prior to immunization, challenge, or skin grafting.

In comparison with the titers in untreated animals, there was a significant delay in the rise in anti-BGG antibody titer in the PHA-treated guinea pigs. Two weeks after immunization, the mean $\log_2$ titer of the untreated guinea pigs was 10, while the titer in PHA-treated animals was 5.5 ($P<.005$). Delayed cutaneous hypersensitivity to intradermal tuberculin challenge was completely suppressed in the PHA-treated animals at the second and third week after immunization. After the three week challenge, PHA was discontinued in the test animals. They were rechallenged again at five weeks, and all developed positive skin reaction. PHA was administered to these animals again for three days commencing two weeks prior to the challenge at seven weeks. None developed a skin reaction to this challenge. Two weeks later, when rechallenged without concomitant PHA administration, all animals again developed positive skin reactions.

Significance to Bio-medical Research and Program of the Institute:

Since the first demonstration of an immunosuppressive action of PHA in 1965, a number of investigators have assessed the effect of this agent on in vivo immune responses to various antigens. Unfortunately, these studies have thus far yielded conflicting results. In individual experiments, PHA both suppressed and enhanced humoral antibody formation. It prolonged skin allograft survival in some experiments, but not in others. Its effect on delayed cutaneous hypersensitivity and as an adjunct to immunosuppressive drug regimens have similarly varied. The incompletely settled question of the effect of PHA on immune responses may be a particularly important one, since PHA has

been administered to humans on many occasions without adverse effects and therefore, could be possibly used clinically as an immunosuppressant. Previous assays of the effect of PHA on immunity were conducted in mice, rats, rabbits, and dogs. Because of the interest in the effect of PHA on cellular immune responses, and the superiority of the guinea pig for such investigations, this animal was chosen for a broad study of the immunosuppressive action of PHA. This first reported assay of the effects of PHA on immune responses in the guinea pig, and first assay of the effect of PHA on a spectrum of immune responses in any test animal, corroborates previous studies in this laboratory and by other investigators which show that PHA is a potent immunosuppressive agent.

Proposed Course of Project:

Publication of data. Future investigations will be explored in the mechanism of suppression of immunity by PHA.

Publications:

Holmes, E.C., Kahan, B.D., and Morton, D.L.: Soluble tumor-specific transplantation antigens from methylcholanthrene-induced guinea pig sarcomas. Cancer 25: 373-379, 1970.

Joseph, W.L. and Morton, D.L.: Immediate function with survival after left lung autotransplantation and contralateral pulmonary artery ligation in the baboon. J. Thorac. Cardiov. Surg. 60: 859-865, 1970.

Joseph, W.L., Melewicz, F., and Morton, D.L.: Experimental lung transplantation in the baboon. J. Surg. Res. 11: 6-11, 1971.

Sparks, F.C., Canty, T., Ting, C.C., Hammond, W.G., and Herberman, R.: Antibody response to skin allografts in mice. Proc. Soc. Exp. Biol. Med. 133: 1392-1396, 1970.

Articles In Press:

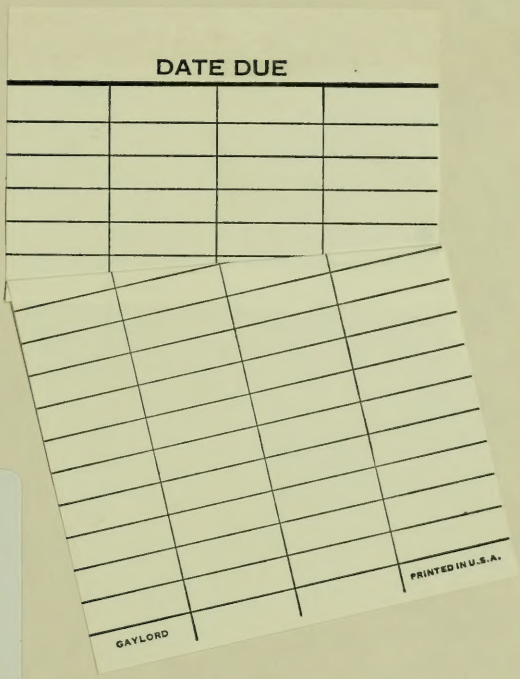
Joseph, W.L. and Morton, D.L.: Long-term survival in the immediately functioning transplanted primate lung: Physiologic findings. Ann. Thorac. Surg.

Joseph, W.L. and Morton, D.L.: Morphologic alterations in the transplanted primate lung. Surg. Gynec. Obstet.

Joseph, W.L. and Morton, D.L.: Influence of ischemia and hypothermia on the ability of the transplanted primate lung to provide immediate and total respiratory support. J. Thorac. Cardiov. Surg.

Joseph, W.L., Melewicz, F., and Morton, D.L.: Delayed cutaneous hypersensitivity to dinitrochlorobenzene as a method of quantitating immunosuppressive therapy for organ transplantation. Surgery

Pilch, B.Z., Gertner, H.R., Sample, W.F., and Chretien, P.B.: Suppression of in vivo immune responses in the guinea pig by phytohemagglutinin. J. Surg. Oncol.



LIBRARY
NIH
Amazing Research.
Amazing Help.
http://nihlibrary.nih.gov

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080

NIH LIBRARY
4 0067 2428

BOUND BY
HM

NIH LIBRARY
3 1496 00185 1149